(51) International Patent Classification 6 : A61K 48/00, C12N 7/01, 15/10, 15/86, C07H 21/04	A1	(11) International Publication Number: **WO 96/36364**
		(43) International Publication Date: 21 November 1996 (21.11.96)

(54) Title: RECOMBINANT VIRAL VECTOR SYSTEM

(57) Abstract

The present invention relates to a system for replication and encapsidation of recombinant DNA fragments into virus particles comprised of adeno-associated viral (AAV) capsid proteins; said system uses a 165 basepair fragment of DNA which contains AAV inverted terminal repeat sequences and which is used to engineer expression vectors useful for gene therapy. The invention provides a means of obtaining recombinant viral stocks that may be used to treat patients suffering from genetic diseases.

RECOMBINANT VIRAL VECTOR SYSTEM

1. INTRODUCTION

The present invention relates to novel
recombinant expression vectors that can be used to
introduce and/or express a heterologous gene of
interest into targeted host cells. The recombinant
expression vectors of the invention contain all the
necessary information required *in cis* for site
specific integration and/or for replication and
encapsidation of the recombinant DNA fragments into
virus particles comprised of adenovirus associated
viral (AAV) capsid proteins. The novel recombinant
expression vectors of the invention provide a means of
transferring genetic information, or alternatively,
for obtaining recombinant viral stocks that may be
used to treat patients suffering from genetic
diseases.

2. BACKGROUND OF THE INVENTION

At the present time, treatments for most
genetic diseases do little to alleviate the symptoms
associated with the genetic disease and considerable
effort is currently underway to develop new, safe and
effective methods of treatment. Recent progress in
the areas of molecular biology and genetic engineering
have lead to the isolation and characterization of
genes associated with genetic diseases. This in turn
has lead to the development of the concept of gene
therapy i.e., the replacement or supplement of
defective genetic information with normal functional
genes, and its potential use for treatment of genetic
disorders.

The most well studied models for gene
therapy involve gene transfer using recombinant
pathogenic viruses to express new genetic information

- 2 -

in order to correct disease phenotypes. Until
recently, the most widely researched viral vectors for
use in gene therapy were the retroviruses (Miller,
A.D., 1990, Human Gene Ther. 1:5-14). One problem
associated with retroviral use is the random
integration of retroviruses into the host genome which
can lead to insertional mutagenesis. In addition, the
long terminal repeats (LTR) structures located at the
ends of the retroviral genome contain promoter/
enhancer activity that may lead to activation of
genetic loci located adjacent to the integrated viral
DNA. For example, integration of retroviral DNA
adjacent to a proto-oncogene may lead to inadvertent
activation of proto-oncogene expression which may, in
turn, lead to transformation and tumorigenesis. This
is illustrated, for example, by recent evidence
indicating that retrovirus vectors in non-human
primates results in T cell lymphomas. More recent
efforts in the field of gene therapy have focussed on
the development of viral vectors lacking the
deleterious characteristics of the retroviruses.

     In addition to the retroviruses, the adeno
associated viruses have also been studied as an
alternative system for delivery of stable genetic
information into the cell. The AAV genome is composed
of a linear single stranded DNA molecule of 4680
nucleotides which contains major open reading frames
coding for the *Rep* (replication) and *Cap* (capsid)
proteins. Flanking the AAV coding regions are the two
145 nucleotide inverted terminal (ITR) repeat
sequences that contain palindromic sequences that can
fold over to form hairpin structures which function as
primers during initiation of DNA replication (FIG. 1).
In addition, the ITR sequences are needed *in cis*, for
viral integration, rescue from the host genome and
encapsidation of viral genomic DNA, into mature

- 3 -

virions (Muzyczka, N. 1992, Current Topics in
Microbiology & Immunology. 158, 97-129).

AAV can assume one of two pathways upon
infection into the host cell. In the presence of
helper virus, AAV will enter the lytic cycle whereby
5   the viral genome is transcribed, replicated, and
encapsidated into newly formed viral particles. In the
absence of helper virus function, the AAV genome
integrates as a provirus into of the host cell genome
through recombination between the AAV termini and host
10   cell sequences (Cheung, A. et al., 1980, J. Virol.
33:739-748; Berns, K.I. et al., 1982, in Virus
Persistence, eds. Mahey, B.W.J., et al. (Cambridge
Univ. Press, Cambridge), pp. 249-265).

Characterization of the proviral integration
15   site and analysis of flanking cellular sequences
indicates that AAV viral DNA integrates specifically
into the long arm of human chromosome 19 (Kotin, R.M.
et al., 1990, Proc. Natl. Acad. Sci. USA 87:2211-2215;
Samulski, R.J. et al., 1991, EMBO J. 10:3941-3950).
20   This particular feature of AAV reduces the likelihood
of insertional mutagenesis resulting from random
integration of viral vector DNA into the coding region
of a host gene. Furthermore, in contrast to the
retroviral LTR sequences, the AAV ITR (inverted
25   terminal repeat) sequences appear to be devoid of
transcriptional regulatory elements, reducing the risk
of insertional activation of proto oncogenes.

Recent work with AAV has been facilitated by
the discovery that AAV sequences cloned into
30   prokaryotic vectors are infectious (Samulski, et al.
1982, Proc. Natl. Acad. Sci. U.S.A. 79:2077-2081).
For example, when a plasmid containing an intact AAV
genome is transfected into cells in the presence of
helper virus, AAV can be rescued out from the plasmid
35   vector and enter the lytic pathway leading to
production of mature virions. In the absence of

- 4 -

helper virus the recombinant AAV vector will integrate
into the host cell genome and remain as a provirus
until the cell subsequently becomes infected with a
helper virus.

     One problem associated with the use of
vectors containing an intact AAV genome is the size
limitations imposed on the vectors by the packaging of
DNA fragments into mature viral particles. Prior to
the present invention it was unknown what viral
sequences were required for replication, and
encapsidation of the DNA into viral particles, or
alternatively, for integration of vector DNA into the
host genome. The present invention defines a novel
165 bp sequences derived from AAV that can function to
direct the replication and encapsidation of DNA into
viral particles and/or the integration of vector DNA
into the targeted host genome.

3. SUMMARY OF THE INVENTION

     The present invention relates to the
in vitro synthesis of a novel 165 basepair fragment of
DNA which contains AAV ITR sequences and which can be
synthesized *in vitro* and used to engineer expression
vectors and/or vectors useful for genetic therapy.
This 165 bp DNA sequence, herein referred to as the
"double-D sequence," is in a novel configuration not
found to exist in wild type AAV.

     The invention is based, in part, on the
ability of the double-D sequence to provide sufficient
information, *in cis*, for converting a circular duplex
DNA molecule into a linear replicating molecule with
covalently closed ends. The replicating DNA molecule
may be either encapsidated into mature AAV virions, or
integrated into the host genome. A particularly
significant feature of the circular duplex DNA
molecule is that the process of conversion into a
linear molecule, and the replication and integration

- 5 -

into the host genome is completely effected through
the double-D sequences, thus ensuring that the
heterologous gene sequences of interest remain intact.

The discovery that the majority of the AAV
genome is not needed, in cis, for replication,
5   packaging and/or integration allows one to insert
larger fragments of DNA into the recombinant vectors.
In addition, the discovery that the 165 double-D
sequence is sufficient for targeting vector DNA to
chromosome 19 allows the insertion of any sized DNA
10   fragment into the recombinant vectors, thereby
removing any size restraints.

Nucleotide sequences which may be used in
accordance with the invention include derivatives and
analogs of the double-D sequences disclosed herein
15   that are functionally equivalent in that they retain
their ability to provide information, in cis, for
replication encapsidation, integration and rescue of
recombinant DNA. In particular, double-D derivatives
may contain additions, substitutions or deletions of
20   the double-D nucleotide sequences while retaining
their biological function.

The invention provides an in vivo system for
replication and packaging of recombinant DNA into
mature virions. Viral stocks containing the
25   recombinant DNA encapsidated into mature virions, may
be obtained by transfection of expression vectors into
a host cell expressing helper function. The resulting
recombinant viral stocks afford a convenient and
efficient means for transfer of genetic information
30   into any cell or tissue of choice.

Alternatively, the double-D constructs may
be delivered into host cells using a variety of DNA
transfer methods such as electroporation, DEAE-
dextran, DNA gun, liposomes etc. One particular
35   advantage associated with these delivery methods is

- 6 -

the absence of size restraints on the size of the
recombinant vectors.

The transfer of genetic information into
host cells, using either viral stocks or transfection
of recombinant constructs, may have applications in
gene therapy wherein the desired goal is correction of
a given genetic abnormality through expression of the
normal complement of the defective gene.

The invention also relates to *in vitro*
treatment of recombinant viral vectors and/or vectors
useful for genetic therapy with bacterial gamma delta
resolvase prior to transfection. Genetic engineering
of resolvase recognition sequences into recombinant
vectors creates the option of removing bacterial
plasmid sequences which would normally be included as
part of the linear, replicated, and encapsidated DNA
molecule. Removal of these bacterial sequences, prior
to replication and encapsidation, allows the maximum
amount of space for insertion of foreign DNA sequences
of interest into the expression vectors.

The invention is described by way of
examples in which an AAV double-D fragment is
amplified in a polymerase chain reaction (PCR) and
inserted into a plasmid vector. Transfection and site
specific integration of these double-D recombinant
vectors into the host genome indicates that double-D
sequences, in the presence of viral REP protein, are
sufficient to direct site specific integration of
vector DNA. In addition, plasmid vectors containing
the double-D sequences were capable of being
replicated and assembled into viral particles. Thus,
the present invention provides a method for
encapsidating recombinant DNA into viral particles
that may be used for gene therapy.

- 7 -

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1.   AAV rescue and replication mechanisms.   (A) Inverted terminal repeats (ITR) may fold back through the base-pairing of A,A', B,B',C,C' sequences to form a T-shape structure.   (B) Excision of infectious plasmid at the ITR sites yields two linear DNA fragments : AAV and the plasmid vector. (C) Predicted fragments generated from double D plasmid after rescue from plasmid circular form to linear with covalent closed ends.

FIG. 2.   Rescue Intermediate from circular double-D plasmid.

FIG. 3.   Replication assay for the plasmid containing the double-D inverted terminal repeat. Plasmid pDD-2 was transfected into Ad5 infected 293 cells with or without cotransfection of the helper plasmid pAAV/Ad.   Low molecular weight DNA was extracted 48 hrs. post infection and separated on a 1% agarose gel with or without DpnI digestion.   (A)-Ethidium bromide staining of the gel.   (B)Southern blot with ^{32}P labeled plasmid pGEM 3z probe.

FIG. 4.   Comparison of replication of pDD-2 with psub201 and pSM620.   Plasmid pDD-2 was cotransfected with equal amounts of either pAAV/Ad (lane 1), psub201 (lane 2) or pSM620 (lane 3) into Ad5 infected 293 cells.   Low molecular weight DNA was extracted and digested with DpnI and analyzed on a 1% agarose gel.   (A) Ethidium bromide staining.   (B) Southern blot with an ITR oligonucleotide probe.

FIG. 5.   Replication assay for double-D plasmids with different sizes.   pDD-2 or pDD-neo were cotransfected with helper pAAV/Ad into Ad5 infected 293 cells.   Low molecular weight DNA was extracted and digested with DpnI and analyzed on a 1% agarose gel. (A)Ethidium bromide staining.   (B)Southern blot with ^{32}P labeled plasmid pGEM 3Z probe.

FIG. 6.   Restriction analysis of rescue and replication of pDD-2. Plasmid pDD-2 was transfected into Ad5 infected or uninfected 293 cells with or without the cotransfection of helper plasmid pAAV/Ad. Low-molecular-weight DNA was digested with either SspI or ScaI and separated on a 1% agarose gel. Southern blot was done with a ^{32}P labeled ITR probe.

FIG. 7.   Cloning of PCR-amplified and EcoRI cut double-D into EcoRI site of plasmid pGEM-3'Z.

FIG. 8.   *In-vitro* replication of parental plasmid containing resolvase sequences.

FIG. 9.   Double-D sequence (SEQ ID NO: 1).

FIG. 10.   Bacterial Gamma Delta Resolvase recognition sequence (SEQ ID NO: 2).

FIG. 11.   PCR amplification of DD-cellular junctions. Genomic DNA was isolated from pooled FACS-sorted 293 cells that has been transfected by different combination of CMV-nLacZ, DD-nLacZ and HIV-Rep plasmid. The first round PCR was performed with JUS3A and RI-Left, the second round PCR reaction was carried out with CR2 and RI-left primers. The Southern blot was probed with a ^{32}P labelled BamHI fragment corresponding to a region chromosome 19.

FIG. 12   Southern blot analysis of the integrated plasmids in FACS sorted 293 genomic DNA. Each lane contains 10 μg of DNA digested by ScaI, PstI and KpnI. The DNA was fractionated on 7% gel and the probe was made from DD-nLacZ plasmid. Lane 1 to 6, 293 cells transfected by DD-nLacZ plasmid, DD-nLacZ plasmid+Rep, DD-nLacZ plasmid+HIV-Rep, CMV-nLacZ plasmid, CMV-nLacZ plasmid+REP, CMV-nLacZ plasmid+HIV-Rep.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to *in vitro* construction of a novel modified AAV ITR nucleotide sequences, herein referred to as the double-D

- 9 -

sequence. The invention is based, in part, on the
synthesis of a double-D DNA fragment using PCR
technology, and the demonstration that this fragment
provides sufficient information *in cis* for replication
and encapsidation of recombinant DNA fragments into
mature AAV virions, or the specific integration of

5   recombinant DNA into the host genome. The recombinant
DNA fragments need not encode any viral proteins
thereby providing a safe method for the transfer of
genetic information into the host genome.

        The invention is based on the discovery that

10   the *cis*-acting double-D sequences, in conjunction with
trans-acting AAV REP protein, are sufficient for site
specific integration of recombinant DNA fragments into
chromosome 19 of the host genome. The invention
relates to the use of the double-D DNA sequences in

15   recombinant vectors and the use of these vectors for
gene replacement therapies.

        The double-D sequences may be genetically
engineered into vectors designed to introduce and/or
express a heterologous gene of interest. For use in

20   gene therapy, the heterologous gene of interest may
represent the normal or wild-type version of a
defective or mutant gene associated with a given
genetic disease. The recombinant vectors, in addition
to containing the coding region for the gene of

25   interest and the double-D sequences, may contain other
necessary regulatory elements such as promoter/
enhancer elements to drive expression of the gene
product in the host, and translation and
polyadenylation signals. The selection of promoter

30   and enhancer regions will rely on the desired level
and tissue specific expression of the gene of
interest.

        The genetically engineered vectors
containing the double-D sequences can be transfected

35   into host cells utilizing any of the techniques

- 10 -

frequently employed by those skilled in the art for
introducing DNA into mammalian cells. For example,
methods including but not limited to electroporation,
DEAE-dextran mediated DNA transfer, DNA guns,
liposomes, direct injection, etc. may be utilized to
5   transfer recombinant vectors into host cells.
Alternatively, the DNA may be transferred into cells
through conjugation to proteins that are normally
targeted to the inside of a cell. For example, the
DNA may be conjugated to viral proteins that normally
10 target viral particles into the targeted host cell.

Host cells may be transfected *in vivo* or
in vitro. For example, cells such as bone marrow
cells, may be removed from the host, transfected with
the recombinant double-D vectors and returned to the
15 host. Alternatively, recombinant double-D vectors may
be encapsulated into liposomes for *in vivo* transfer of
genetic information into host cells.

One significant advantage associated with
transfection of double-D vectors into host cells is
20 the absence of restraints on the size of the
recombinant vectors. This is in contrast to the size
limitations imposed by the packaging of DNA fragments
into mature virus particles. Another advantage is
that there is no risk of generating infectious viral
25 particles when the treated individual later becomes
infected with helper virus and AAV.

The recombinant vectors may also be
transfected into a host cell line that provides helper
virus function and supplies *in trans* the *REP* and *CAP*
30 proteins allowing one to obtain a recombinant virus
stock (Muzyczka, N. 1992, Current Topics in
Microbiology and Immunology 158:97-129). The
resulting virus stock may then be used as a highly
efficient means of delivery and transfer of new
35 genetic information into targeted cells or tissues of
choice.

5.1. THE DOUBLE-D SEQUENCES

The AAV genome consists of 4680 nucleotides containing two open reading frames that encode the *REP* (replication) and *CAP* (capsid) proteins. Located at both ends of the genome are 145 bp inverted terminal repeats (ITRs), which are unique in that they not only basepair with each other, but also individually fold back on themselves through the basepairing of A, A', B, B', C, C' sequences to form a T-shaped structure for DNA replication when single stranded (FIG. 1A).

When a plasmid containing an intact AAV genome is transfected into helpervirus infected cells, AAV can be rescued or replicated out from the plasmid vector and enter the viral lytic cycle leading to production of mature virions. If the AAV coding region is deleted and replaced by heterologous DNA sequences, the recombinant AAV can still complete the viral lytic cycle provided the ITRs are intact and the *REP* and *CAP* proteins, or functional equivalents, are supplied *in trans*. However, if one of the two ITR sequences are deleted no viral DNA replication is observed indicating that both ITRs are required for AAV viability.

The invention is based, in part, on the discovery that the following 20 basepair D sequence (AGGAACCCCTAGTGATGGAG) (SEQ ID NO: 5), present in the ITR sequence I required for viral replication. This is initially demonstrated by the inability of viral mutants with deleted D sequences to replicate their DNA Furthermore, during the replication of a terminal resolution site mutant, natural deletions were found to occur only towards the A sequence of the ITR and not towards the D end, suggesting a selection process for retention of D sequences.

In order to elucidate further the function of the D sequences, a novel modified terminal repeat structure was constructed containing a single 145 bp

- 12 -

ITR sequence with an additional 20 bp D' sequence
(FIG. 9) (SEQ ID NO: 1) (See Section 6.13, _infra_).
The resulting 165 bp sequence has not been identified
in any naturally occurring virus. Using AAV DNA as
template and a single primer derived from the D
5 sequence of the AAV ITR sequence plus a 6bp EcoRI
recognition site on the 5' end, a polymerase chain
reaction was performed that resulted in a DNA fragment
comprised of an ITR flanked on either side by D or D'
sequences as well as EcoRI sites. The PCR generated
10 DNA fragment was cleaved by EcoRI and subsequently·
cloned into the EcoRI site of pGEM3Z (FIG. 7).

          To determine whether the double-D was able
to function in replication, encapsidation, integration
and rescue of recombinant DNA, a recombinant plasmid
15 containing the double-D structure was transfected into
cells . Results from these experiments indicate that
the novel double-D sequence is sufficient to carry out
the functions normally required of two wild type ITRs
during a lytic AAV viral infection.

          In addition, cotransfection of recombinant
20 DNA containing double-D sequences and DNA encoding AAV
REP protein indicate that the double-D sequences and
the _REP_ protein are all that is required for site
specific integration into the host genome. (See
Section 6.2.3. and Section 6.2.4., _infra_). Analysis
25 of the viral integration site indicates that
integration takes place through the ITR sequences,
thus ensuring that the vector sequences, i.e. those
sequences containing heterologous sequences of
interest remain intact.
30
          In addition to the double-D sequence of FIG.
9 (SEQ. ID NO.: 1), nucleotide sequences capable of
hybridizing to the double-D sequences (SEQ ID NO:1)
under highly or less highly stringent hybridization
conditions are well within the scope of the invention.
35 Highly stringent hybridization conditions may be

- 13 -

defined as hybridization to filter-bound DNA in 0.5 M
NaHPO4, 7% sodium dodecyl sulfate (SDS), 1mM EDTA at
65°C, followed by washing in 0.1 x SSC/0.1% SDS at
68°C (Ausubel F.M. et al., eds, 1989, Current
Protocols in Molecular Biology, Vol. I, Green
Publishing Associates, Inc., and John Wiley & Sons,
Inc., New York at p. 2.10.3). Less highly stringent
conditions, such as moderately stringent conditions,
may be defined as hybridizations carried out as
described above, followed by washing in 0.2x SSC/0.1%
SDS at 42°C (Ausubel et al., 1989, supra).

In an additional embodiment of the
invention, double-D sequences from other adenovirus-
associated viral subtypes which form hairpin
structures, may be used for isolating double-D DNA
fragments. Alternatively, altered nucleotide
sequences which may be used in accordance with the
invention include derivatives and analogs of the
double-D sequence that are functionally equivalent in
that they retain their ability to provide information,
in cis, for replication, encapsidation, integration
and rescue of recombinant DNA. In particular,
double-D derivatives may contain additions,
substitutions or deletions of nucleotide sequences but
still retain biological function.

The present invention includes methods for
identifying additions, substitutions or deletions of
double-D sequences that retain double-D function.
Alterations in the double-D sequences may be generated
using a variety of chemical and enzymatic methods
which are well known to those skilled in the art. For
example, oligonucleotide-directed mutagenesis may be
employed to alter the double-D DNA sequence in a
defined way and/or to introduce restriction sites in
specific regions within the double-D sequence.
Alternatively, deletion mutants may be generated using
DNA nucleases such as Bal 31 or Exo III and S1

- 14 -

nuclease. Progressively larger deletions in the
double-D sequences may be generated by incubating said
DNA with nucleases for increased periods of time.
(See Ausubel, et al., 1989 Current Protocols for
Molecular Biology, for a review of mutagenesis
techniques).
5

The altered double-D sequences may be
evaluated for their ability to provide information,
in cis, for replication, encapsidation, integration
and rescue of recombinant DNA using, for example, any
of the methods described herein (See e.g., Section 6
10
supra). It is well within the scope of the present
invention that any altered double-D sequences that
retain their ability to provide information, in cis,
for replication, encapsidation, integration and rescue
of recombinant DNA may be incorporated into
15
recombinant expression vectors which may then be
employed for transfer of genetic information into host
cells.

A number of advantages are associated with
the use of recombinant vectors containing double-D
20
sequences. One advantage is that the double-D
sequences enable these vectors to convert from
circular duplex molecules into linear replicating
molecules with covalently closed hairpin ends (see
Figure 6). This is believed to be essential as the
25
formation of linear molecules is a major prerequisite
for successful integration of recombinant DNA into the
host genome. The spontaneous formation of linear
molecules, in the absence of any viral proteins,
represents a significant advantage over the use of
30
other AAV based vectors which require linearization
prior to introduction by transfection into host cells.
Additionally, the formation of linear
replicating molecules and the integration of the
replicated molecules via the double-D sequences
35

- 15 -

ensures that the DNA sequences ensures that the DNA
requires encoding the gene of interest remain intact.

5.2.   CONSTRUCTION OF RECOMBINANT VECTORS
COMPRISED OF DOUBLE-D SEQUENCES
<u>AND HETEROLOGOUS LINKED SEQUENCES</u>

5   The double-D sequences (SEQ ID NO: 1) of the
invention provide all the necessary information for
directing the replication and encapsidation of
recombinant DNA into mature virions. A DNA fragment
containing a double-D nucleotide sequence may be
10   obtained by any number of methods commonly used in the
art. In a specific embodiment, described herein, a
polymerase chain reaction (PCR) was used to obtain a
double-D DNA fragment using AAV DNA as template and a
primer derived from the D sequence of the AAV ITR.
15   The rationale for this approach is based on the
expected secondary structure of the natural ITR
sequence.

In the first round of the PCR reaction, the
AAV viral ITR forms a hairpin structure and self-
20   primes the elongation process to produce a long
T-shaped hairpin structure containing D and D' on the
stem. Upon denaturation, this DNA serves as template
for a single-primed PCR reaction. Alternative methods
for isolating a double-D DNA fragment, include but are
25   not limited to chemically synthesizing the DNA
sequence.

Standard recombinant DNA methods may be used
for insertion of novel double-D sequences into
recombinant vectors which may include, for example,
30   plasmid or cosmid vectors. The methods include *in
vitro* recombinant DNA techniques, synthetic techniques
and *in vivo* recombination/genetic recombination. For
example, the double-D DNA sequence may be amplified in
a PCR reaction using oligonucleotide primers that add
35   an appropriate restriction endonuclease recognition

- 16 -

site onto each end of the amplified DNA fragment (See
Section 6.1.3). Alternatively, any restriction site
desired may be produced by ligating nucleotide
sequences (linkers), comprising specific chemically
synthesized oligonucleotides encoding restriction
endonuclease recognition sequences, onto the termini
of the amplified double-D fragment into an expression
vector having complementary cohesive termini.

A variety of host recombinant vector systems
may be utilized equally well by those skilled in the
art. The recombinant vectors could contain bacterial
plasmid sequences necessary for replication in *E.coli*
or the *cis* acting double-D sequence could be ligated
directly to the gene of interest. In addition,
plasmids will contain DNA sequences coding for a
heterologous gene of interest, inserted between the
appropriate transcriptional/translational control
sequences and polyadenylation signals. A variety of
promoter/enhancer elements may be used depending on
the level and tissue specific expression desired.
Promoters produced by recombinant DNA or synthetic
techniques may be used to provide for transcription of
the inserted gene of interest. Specific initiation
signals are also required for efficient translation
of inserted protein coding sequences. These sequences
include the ATG initiation codon and adjacent
sequences. In addition, polyadenlylation signals may
be included to increase the stability of transcribed
mRNA.

One potential drawback of the AAV viral
vector system is the size limitation imposed by the
inability of DNA fragments larger than 5 Kb to be
packaged into mature virus particles. In any given
expression vector, 2-3 Kb of DNA sequence derives from
bacterial plasmid sequences which are required for
propagation of plasmid in *E. coli*. These DNA
sequences include origin of replication (*ori*)

sequences and genes that confer resistance to
antibiotics such as ampicillin and tetracycline. In
effect, this leaves only 2 Kb of space for insertion
of heterologous genes of interest.

The following particular, non-limiting
embodiment of the invention addresses this size
limitation problem. To increase the amount of space
available for cloning of sequences of interest, a
bacterial recombination system (gamma delta) may be
used to resolve plasmids in such a manner that the
majority of the bacterial plasmid DNA sequences will
be recombined out of any given recombinant plasmid
construct *in vitro*, thereby allowing for the maximum
amount of space for insertion of foreign genes. The
gamma delta resolvase sequences may be used in a
variety of viral vector systems, not limited to AAV
systems, as a general method for increasing the space
available for insertion of foreign genes.

The parental double-D expression vector
plasmid may be engineered to contain, in addition to
the double-D sequence, two copies of the gamma delta
resolution site (FIG. 10) (SEQ ID NO: 2) (120
basepairs) in the correct orientation so as to promote
recombination when resolved *in vitro* with gamma delta
resolvase enzyme. In the presence of gamma delta
resolvase, the recombinant plasmid should be converted
into two circular DNA molecules (FIG. 8). One plasmid
molecule should contain the primary bacterial plasmid
sequences along with a copy of the gamma delta
resolution site. The other resolved plasmid molecule
would be expected to contain the double-D *cis* acting
sequences, one copy of a gamma delta resolution site
and the coding region for the gene of interest. It is
this plasmid molecule which will be converted, in the
presence of helper virus and the viral *REP* and *CAP*
proteins, into a linear replicating molecule which
will then be encapsidated into mature viral particles.

- 18 -

Currently, a second plasmid is required to
supply the *CAP* and *REP* proteins which are needed to
convert circular plasmid molecules into replicating
linear DNA fragments. In a further particular
embodiment of the invention, plasmids containing the
gamma delta resolution sites may be engineered to also
contain the coding regions for the required viral *REP*
and *CAP* functions. Normally the *REP* and *CAP* coding
sequences would be excluded from the expression vector
because they further limit the size of your insert.
Using the *in vitro* recombination system, these coding
regions may be included in the plasmid construct,
since they will be recombined out during resolution
through the two gamma delta resolution sites by the
resolvase enzyme (FIG. 8). The products are two
concatenated DNA molecules.

Because the *in vitro* gamma delta reaction is
a linear reaction, the amount of resolved molecules
may be controlled in the *in vitro* resolving reaction
to generate desired ratios of parental plasmid to
resolved circles. The mixture of plasmids can then be
transfected into the host cell. This introduces one
circular molecule containing the AAV *REP* and *CAP* genes
required *in trans* for a productive replication, and
the other circular molecule contains the double-D
sequences and the gene of interest. The double-D
circular molecule may be replicated into a linear DNA
molecule which may subsequently be encapsidated into
viral particles by the *trans* factors introduced on the
REP/CAP circular plasmid.

The present invention further provides for
the analogous use of the above-described gamma delta
resolvase system to first propagate plasmids that
comprise (i) a recombinant viral vector sequence
comprising a gene of interest, (ii) viral genes
providing helper functions, and (iii) two gamma delta
resolvase recognition sequences flanking the viral

vector sequence (SEQ ID NO: 2) and, second, to
separate recombinant viral vector sequences comprising
the gene of interest from the remaining plasmid
sequence using resolvase enzyme. According to these
methods, the virus from which the vector and helper
functions are derived may be any suitable virus
including but not limited to AAV, a retrovirus,
adenovirus, or herpes virus. In preferred embodiments
the viral vector portion of the plasmid comprises the
double-D sequence or, alternatively, both AAV ITR's.
In general, the viral vector protein comprises
sequences necessary for encapsidation and
transcription of the gene of interest.

5.3. PRODUCTION OF RECOMBINANT VIRUS STOCKS

The invention relates to a method for
replicating and encapsidating a recombinant DNA
molecule into an AAV particle which comprises
culturing a eukaryotic cell containing helper virus,
recombinant DNA encoding AAV *REP* and *CAP* proteins, and
a recombinant nucleic acid containing a DNA sequence
of interest and the 165 base pair double-D sequence.

To generate recombinant viral stocks, the
double-D recombinant expression vector plasmid may be
transfected into a host cell line that is capable of
providing helper virus function, and supplying *in
trans* AAV *REP* and *CAP* proteins. The *REP* and *CAP*
proteins are required for replication and
encapsidation of the linear recombinant DNA into
mature viral particles.

The *REP* and *CAP* proteins may be supplied *in
trans* by transfection of the host cell line with a
recombinant plasmid that is capable of coding for each
of the proteins. DNA transfections may be carried out
using methods well known to those skilled in the art.
These may include DNA transfection by lipofection,
electroporation or calcium phosphate precipitation

- 20 -

(Ausubel, et al., 1989, in Current Protocols for
Molecular Biology, Green Publishing Associates, Inc.
and John Wiley & Sons, Inc., New York). The plasmid
is transfected into the host cell line with the
intention of either transiently or stably expressing
the *REP* and *CAP* proteins. In a specific embodiment,
described in Section 6.1., the plasmid pAAV/AD
containing the AAV coding regions, was transfected
into a host cell for the purpose of expressing the *REP*
and *CAP* proteins.

In another embodiment, the double-D
expression vector may be engineered to directly
express the *REP* and *CAP* proteins. In this case, it is
also important to include the gamma delta resolvase
sequences in the plasmid vector, so that the *REP* and
CAP coding regions may be recombined out during an *in
vitro* resolvase reaction so as not to impose a size
limitation on the insert of foreign DNA.

In addition to expressing the viral *REP* and
CAP proteins, the host cell lines must be able to
provide helper virus function. Both adenovirus and
herpes simplex virus may serve as helper viruses for
replication of DNA fragments containing the double-D
sequences. Any host cell permissive for infection by
either of these two viruses or any virus that acts as
a helper virus for AAV, may be used in the practice of
the invention. The multiplicity of infection (MOI)
and the duration of the infection time will depend on
the type of virus used and the cell line employed.

In a specific embodiment, described herein,
293 cells which had previously been transfected with a
recombinant double-D expression vector, were infected
with Ad5 at a MOI of 10. Forty-eight hours later the
cells were frozen and thawed three times, and
incubated for one hour at 56°C to inactivate the
adenovirus. The resulting cell lysate contains

recombinant viral particles that may be used to infect
cells or tissue of choice.

Alternatively, the recombinant double-D DNA
vectors may be grown up and purified for use in DNA
transfections, using any of the methods well known to
those skilled in the art. (See *Sambrook et al.*, 1989,
Molecular Cloning: A Laboratory Manual, Cold Spring
Harbor Laboratory Press, Cold Spring Harbor, N.Y., for
description of methods that may be used to propagate
recombinant vector DNA.)

5.4. USES OF RECOMBINANT VECTORS

The double-D expression vectors, containing
a heterologous gene of interest and described herein,
may be useful for therapeutic treatment of genetic
disorders. The gene of interest may be the wild type
complement of any mutated or defective gene and may be
inserted into the double-D recombinant vectors so that
its expression is controlled by its natural promoter
(*e.g.*, so that expression is regulated normally) or by
a heterologous promoter.

The double-D expression vectors may be
transfected into host cells using any of the
techniques routinely used by the skilled artisan for
efficient transfer of nucleic acids into host cells.
Host cells may be transfected *in vivo* or *in vitro*.
For example, cells may be removed from the host and
transfected with recombinant vectors. The transfected
cells may be assayed for proper integration of the
recombinant DNA into chromosome 19 of the host. The
cells may also be tested to determine whether the gene
product of interest is properly expressed. Following
the verification of proper integration and expression
of the recombinant DNA, the transfected cells may be
implanted or returned into the host.

Alternatively, transfer of recombinant DNA
may take place *in vivo*. In a preferred embodiment of

- 22 -

the invention the recombinant double-D vectors may be encapsulated into liposomes for delivery into host cells. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules that are present in an aqueous solution at the time of liposome formation

5   (in this case, recombinant vectors and/or REP protein) are incorporated into this aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into

10   the cell cytoplasm. The double-D vectors of the invention, may be co-encapsulated into liposomes with viral *REP* protein, RNA molecules encoding *REP* protein or viral DNA sequences encoding the *REP* protein, to provide for site specific integration of viral vector

15   sequences into the host chromosome.

Additionally, recombinant viral stock may be generated by transfection of the double-D plasmid into a suitable host cell line that first allows for conversion of a circular duplex molecule into a linear

20   DNA molecule covalently closed at both ends. This then permits replication and encapsidation of recombinant DNA into mature viral particles. The resulting viral stocks may then be used to infect tissue or cells affected by the genetic defect.

25
         6.   EXAMPLE: A NOVEL 165 BASE PAIR TERMINAL
              REPEAT IS THE ONLY *cis*-ELEMENT REQUIRED
              FOR ADENO-ASSOCIATED VIRUS LIFE CYCLE

         The subsection below describes the synthesis and functional characterization of the double-D

30   sequence.

              6.1. MATERIALS AND METHODS
              6.1.1. DNA TRANSFECTION
         Human cell line 293 was maintained in DMEM

35   (Dulbecco modified Eagle medium, GIBCO) WITH 10% FCS
     (fetal calf serum, HyClone). Transfection of plasmid

- 23 -

DNA was done by lipofection (BRL) method as described
by the manufacturer. Briefly, cells in a 6-cm dish
were washed twice with DMEM and infected with
Adenovirus 5 at 10 moi (multiplicity of infection) in
1ml Opti-MEM (GIBCO) for 1 hr. Then 5 ug plasmid DNA

5   was incubated with 50 ul of lipofectin (BRL) at room
temperature for 10 min, mixed with 2 ml of Opti-MEM
and added to the Adenovirus infected cells. After
incubation for 12 hrs., the cells were fed with 3 ml
of DMEM containing 4% FCS and incubated for an

10 additional 36 hours.

                6.1.2.   SOUTHERN HYBRIDIZATION
        Low molecular weight DNA from transfected
cells was extracted as described by Hirt (Hirt,

15 B.1967, J. Mol. Biol. 26:365-369). The DNA was
digested by restriction enzymes (New England BioLab),
separated on an agarose gel, then transferred onto the
Genescreen plus Nylon membrane (DuPont).
Hybridization with ^{32}P labeled plasmid DNA was carried

20 out as recommended by the manufacturer. Hybridization
with r- ^{32}P-ATP end-labeled ITR oligonucleotide probe
A-1 (5'TTGGCCACTCCCTCTCTGCG3') (SEQ ID NO: 4), derived
from A region of ITR, kindly provided by N. Muzyczka)
was performed as follows: the membrane was

25 prehybridized in 10 ml solution containing 5X SSC, 10X
Denhardt's solution, 10% dextran sulfate and 5% SDS at
60°C for at least 1 hr. 25 ng of ^{32}P end labeled
oligo-probe and 200 ug heat-denatured salmon sperm DNA
in 0.5 ml H$_2$O were added. Hybridization was continued

30 at 60°C overnight. The membrane was washed twice in
3X SSC and 5% SDS at 60°C for 30 minutes and once in
0.2X SSC at room temperature for 10 minutes. For
Southern blot analysis of lacZ expressing cell clones
a ^{32}P labeled chromosome 19 specific probe was used

35 (Samulski, R.J. et al., 1991, EMBO J. 10:3941-3950).
        To determine whether the CMV/lacZ DNA

- 24 -

fragments had integrated specifically into chromosome
19, high molecular weight genomic DNA was extracted
from transfected cells as described by Sambrook et al.
(1989, Molecular Cloning: A Laboratory Manual. Cold
Spring Harbor Laboratory Press. Cold Spring Harbor,
N.Y.). The genomic DNA was used as template in PCR
reactions designed to amplify junction fragments. The
following PCR primer was used in the PCR reaction.

        5'-GTGAATTGTAATACGACTCACTATAGGGCG-3'.

10   6.1.3.   PCR AND CONSTRUCTION OF ITR PLASMID
            Low molecular weight DNA from AAV and Ad5
infected cells was used as template for the PCR
reaction with a single primer derived from D-sequence
of AAV. The PCR was performed at 94°C 1 min., 45°C 30
seconds and 72°C 1 min. for 35 cycles in a 50 ul
reaction solution containing 20 mM Tris-HCl (pH8.8),
1.5 mM MgCl, 50 mM KCl, 2.5% formamide, 100 uM dATP,
dCTP and dTTP, 75 uM 7-deazo-dGTP, 25 uM dGTP, 1.5U
AmpliTaq (Perkin Elmer Cetus), 1 ng AAV DNA and 100
pmole primer TR-1 (5'-GGAATTCAGGAACCCCTAGTGATGG3-3')
(SEQ ID NO: 3). The PCR product was purified by
agarose gel electrophoresis, cut with EcoRI and
ligated with an EcoRI cut and dephosphorylated pGEM 3Z
plasmid (Promega). The ligated plasmid was
transformed into E.coli Sure strain (Stratagene).
Positive clones named pDD's were screened for the
presence of double-D terminal repeat and confirmed by
dideoxy-sequencing with 7-deazo-dGTP substituted for
dGTP (Sanger, F. et al., 1977, Proc. Natl. Acad. Sci.
USA 74:5463-5467). Subsequently, a neo gene was
cloned into the SalI site of pDD-2 resulting in the
plasmid pDD-neo.

        6.1.4.   CLONING OF NEO-RESISTANT CELL LINES
            Ad5 infected 293 cells were cotransfected
with pDD-neo and pAAV/Ad (Samulski, et.al., 1989. J.

Virol. 63:3822-3828) for 48 hrs. The cells were
frozen and thawed three times and then incubated at
56°C for 1 hour to inactivate the Ad5 virus. The cell
lysate containing the DD-neo recombinant AAV virus was
used to infect the human cell line Detroit 6. The

5   cells were inoculated for 24 hours, then selected with
G418 at 400ug/ml to obtain neo-resistant clones.
Various clones were superinfected with wild-type AAV
and Ad5 at a MOI of 10 to rescue the latent neo-AAV.

6.2.   RESULTS

10   6.2.1.   CONSTRUCTION OF ITR WITH DOUBLE D SEQUENCE

The Polymerase Chain Reaction (PCR) was used
to construct the inverted terminal repeat with a D'
sequence added to the other end. The rationale is
based on the T-shape structure of the ITR. In the

15   first round of PCR reaction, the AAV viral IRT will
self-prime the elongation to produce a long T-shaped
hairpin structure containing D and D' on the stem.
Upon denaturation, this DNA can serve as template for
single-primed PCR.

20   Because of the high GC content and the
strong palindromic structure in the ITR region,
several strategies such as 7-deazo-dGTP, 2.5%
formamide, and high concentration of primer were
utilized to tackle the PCR problems and yield

25   sufficient desired PCR product. For the convenience
of cloning, an EcoRI recognition sequence was attached
to the 5'of the primer so that the PCR product can be
cut by EcoRI and easily cloned into the polylinker of
pGEM 3Z. Due to the instability of the ITR in

30   bacteria host, the recombinant plasmid was transformed
into an *E.coli* SURE strain (Stratagene) in which the
ITR was rather stable. By using the above strategy,
we obtained numerous positive clones. Some clones
were characterized by restriction digestion and

35   sequencing. One of the clones is shown in Figure 2

- 26 -

bearing an insert of D'ABB'CC'A'D in the EcoRI site of
the pGEM-3Z. This plasmid was named pDD-2 and was
used in the following transfection experiments.

.6.2.2. <u>pDD-2 REPLICATION IS DEPENDENT ON REP</u>

5
In order to assay the capability for
replication, Plasmid pDD-2 was transfected into Ad5
infected 293 cells with or without cotransfection of a
helper plasmid pAAV/Ad, which contains functional REP
and CAP genes but without the ITR, so that it can not
replicate. Due to the lack of functional origins this

10
molecule can only supply REP and CAP proteins in
trans. Post transfection for 48 hours, the plasmid
DNA was extracted and separated on 1% agarose gel with
or without DpnI digestion. DpnI only digests the
input methylated plasmid DNA while leaving the

15
replicated (demethylated) DNA intact. The results
demonstrated that in the absence of the helper
plasmid, pDD-2 plasmid did not replicate therefore the
DNA is completely DpnI sensitive (FIG. 3, lane 1 and
2). However, in the presence of the helper plasmid,

20
pDD-2 replicated very efficiently as evidenced by the
resistance to DpnI digestion and the existence of
monomer and dimer molecules: the typical AAV
replication pattern (FIG. 3, lane 3 and 4). The pDD-2
replication is dependent on two factors: the double-D

25
in cis and REP gene products in trans, because the
cloning vector pGEM-3Z did not replicate under the
same conditions and a plasmid containing only REP gene
without CAP gene can also supply the helper function
in trans for pDD-2 (data not shown).

30
Since the replication of pDD-2 with one
modified ITR was very efficient, a comparison was made
between pDD-2 and two other infectious AAV plasmids,
psub201 [Samulski, et.al., 1987. J. Virol, 61:3096-

35
3101.] and pSM620 [Samulski, et.al., 1982, Proc. Natl.
Acad. Sci. U.S.A. 79:2077-2081.], which possesses two

ITRs as well as wild type *REP* and *CAP* genes. The
pDD-2 was cotransfected into Ad5 infected cells with
equal amounts of either pAAV/Ad helper (without ITR),
psub201 or pSM620. The plasmid DNA was extracted 2
days post transfection, digested with DpnI, separated
on 1% agarose gel. Southern blot was performed with
an oligonucleotide probe from the A sequence of the
ITR so that it can detect all the replicated DNA
containing ITRs. As shown in Figure 4, all three
plasmids containing AAV coding genes can complement
the pDD-2 replication equally well. However, psub201
itself replicated at a much lower level although it
can complement pDD-2 replication effectively. pSM201
replicated at a similar level as pDD-2.

In order to determine whether the
effectiveness of pDD-2 replication was due to the
special double-D or due to the smaller size of the
plasmid (2.9 kb), a neo gene fragment of 1.2 kb was
inserted into the SalI site at the polylinker of
pDD-2. The new plasmid pDD-neo is 4.1 kb in size,
close to the size of wild type AAV (4.68 kb). This
plasmid converted from a duplex circular to a linear
molecule and replicated as efficiently as the parental
pDD-2 (FIG. 5). double-D plasmids were constructed
with sizes up to 7.5 kb . These molecules also
efficiently replicate (data not shown). The above
results suggest that the double-D is an excellent
substrate for Rep-dependent replication.

6.2.3.   REPLICATION AND RESCUE IS VIA AAV MECHANISM
AAV inverted terminal repeats have been
proven to be the viral replication origins. *In vitro*,
these sequences are recognized as a substrate by *REP*
protein, a site-and-strand-specific nickase and
helicase. ITRs have also been considered as the
substrate for AAV rescue [Muzyczka, N. 1992, Current
Topics in Microbiology & Immunology. 158, 97-129].

- 28 -

Since the double-D plasmids contain one unique ITR and
we have demonstrated that this sequence replicates
only in the presence of *REP* proteins, it is attractive
to predict that the rescue and replication are through
similar AAV rescue and replication mechanisms (FIG. 1,
A and B).

5

In order to test the above assumption, pDD-2
DNA was transfected into Ad5 infected 293 cells with
or without helper plasmid, or transfected into
uninfected 293 cells. Subsequently, the plasmid DNA
was subjected to restriction analysis by two single

10

cutter enzymes ScpI and ScaI respectively (for map,
see FIG. 2). The DNA was probed with ITR
oligonucleotide so that only the ITR-containing
fragments would be detected. The results are shown in
Figure 6. After SspI or ScaI digestion, a linear full

15

length plasmid band could be observed throughout all
the lanes (P to 6). This band was derived from the
unresolved input circular plasmid. While in lane 1
and 4 (Ad5 plus helper plasmid), four additional bands
with expected molecular weight could also be seen.

20

Two of them arose from internal head-to-head and tail-
to-tail fragments of the digested dimer molecules..
The other two bands are derived by digested monomer
and dimer external fragments, most likely suggesting
that pDD-2 is resolved at the unique double-D site and

25

replicated via AAV replication scheme. It is
noteworthy that in Ad5 infected cells (lane 2 and 5)
and uninfected cells (lane 3 and 6), two fainter bands
from the resolved monomer were also visible,
suggesting that some cellular mechanism can initiate

30

the rescue process at the double-D site in the absence
of any other AAV sequence or AAV gene product.
Although, such rescued DNA could not replicate in the
absence of Rep proteins (see FIG. 3, lane 2) this
suggests that the double D substrate may confer

35

special features involved in the first step of AAV

recognition not seen with the conventional AAV
plasmids containing two wild type ITR's.

6.2.4.   ONE DOUBLE-D IN-*CIS* IS SUFFICIENT FOR AAV VIABILITY

5   Plasmid pDD-neo was used to generate the
DD-neo virus preparation as described in Section
6.1.4.   The cell lysates containing the recombinant
virus particles were then used to infect human Detroit
6 cells.   Two weeks post infection cells were selected
10 against G418.   A number of neo-resistant clones were
isolated, indicating that the recombinant viruses were
made and transduction was accomplished. DD-neo
resistant cell lines were superinfected with wild type
and AAV-2 and Ad5 and assayed for transduced DNA
15 rescue and replication. Then the viral DNA was
extracted and probed with a neo gene fragment.
Examples of DD-neo'cell lines that rescued DD-neo
viral DNA replicated as monomer and dimer (data not
shown).   These results demonstrated that the 165 bp
20 single double-D is the only *cis*-sequence required to
fulfill all the steps in AAV life cycle.   Thus, the
processes such as rescue from the plasmid, replication
of the DNA, encapsidation of the virus, infection into
the cells, integration into the chromosome and rescue
25 back again were all mediated by this unique double D
inverted terminal repeat sequence.

6.2.5.   TRANSFECTION OF DOUBLE-D RECOMBINANT VECTORS

30   Human 293 cells were transfected with either
double-D ITR CMV/LacZ constructs or CMV/LacZ
constructs lacking the double-D sequences.   These
particular constructs contain the lacZ gene which
encodes the E. coli β-galactosidase reporter protein,
35 under the transcriptional control of the CMV

- 30 -

(cytomegalovirus) constitutive promoter. In addition, the CMV/LacZ plasmids were cotransfected with pHIV-REP, a recombinant plasmid containing the gene encoding AAV REP protein under the transcriptional control of the HIV promoter (Antoni, B.A. et al., 1991, J. Virology 65:396-404).

Six weeks post-transfection cells were stained to determine what percent of the cells were expressing β-galactosidase activity. Detection of β-galactosidase activity in cell extracts indicates the presence of chromosomally integrated lacZ constructs. Only the double-D CMV/LacZ constructs that were cotransfected with a vector capable of expressing the REP protein were found to be expressing β-galactosidase activity.

Southern Blot analysis of the transfected cells confirmed that those cells expressing β-galactosidase activity had integrated the double-D CMV/LacZ constructs into their genome (Fig. 11 and 12). In addition, PCR analysis indicated that the CMV/LacZ plasmids had integrated into the specific region of chromosome 19 into which wild type AAV normally integrates (TABLE I).

- 31 -

TABLE I

<u>Cells: TA55, TA67, TA69:</u>

GTGAATTGTAATACGACTCACTATAGGGCGAATTCAGGAACCCCTAGTGATGGAGT

TGGCCACTCCCTCACTGCGCGCTCGCTCGCTCACTGAGGCCG<u>ACGGTATCAGCGCC</u>

<u>CTGCACCAGGTCAGCGCCCCCCGC</u>

5   AAV sequence is joined to BamHI fragment of chromosome
           19 at position 1695.

<u>Cells: TA8, TA22, TA65, TA76:</u>

GTGAATTGTAATACGACTCACTATAGGGCGAATTCAGGAACCCCTAGTGATGGAGT

TGGCCACTCCCTCACTGCGCGCTCGCTCGCTCACCGCAGCGA<u>GGCCTGGGCTTTGC</u>

10   <u>CACCCTATGGT</u>

AAV sequence is joined to BamHI fragment of chromosome
19 at position 1830.

<u>Cells: TA18, TA29:</u>

GTGAATTGTAATACGACTCACTATAGGGCGATTCAGGAACCCCTAGTGATGGAGTT

GGCCACTCCCTCACTGCGCGCTCGCTCGCTCACTGAXGG<u>CTTTGCCACCCTATGGT</u>

15   <u>GACACCCC</u>

AAV sequence is joined to BamHI fragment of chromosome
19 position 1836.

<u>Cell: TA60, TA68:</u>

GTGAATTGTAATACGACTCACTATAGGGCGATTCAGGAACCCCTAGTGATGGAGTT

20   GGCCAC<u>CCTATGCTGACACCCCGTCCC</u>

AAV sequence is joined to BamHI fragment of chromosome
19 at position 1843.

<u>Cells: TA16:</u>

25   GTGAATTGTAATACGACTCACTATAGGGCGAATTCAGGAACCCCTAGTGATGGAGTT

GGCCACTCCCTCACTGCGCGCTCGCTCGCTCAC<u>CCCCACTTCCGAATTGGAGCCGC</u>

AAV sequence is joined to BamHI fragment of chromosome
19 at position 1891.

<u>Cells: TA45, TA63, TA64, TA70, TA75, TA88:</u>

30   GTGAATTGTAATACGACTCACTATAGGGCGAATTCAGGAACCCCTAGTGATGGAGT

TGGCCACTCCCTCACTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTT

CGCCGACGCCCGGGCTTTGCCC<u>TCTCCTGAACCTGAG</u>

AAV sequence is joined to chromosome 19 at position
<u>2143.</u>

Sequences of Chromosome 19 are underlined.

35

- 32 -

These data indicate the following: (i) that only *cis*-acting sequence required for targeted integration by AAV is the double-D sequence and (ii) the AAV REP protein is sufficient to act *in trans* to target site specific integration of recombinant vector
5 DNA into genomic host DNA.

The present invention is not to be limited in scope by the exemplified embodiments disclosed herein which are intended as illustrations of single aspects of the invention, and any clones, DNA or amino
10 acid sequences which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing
15 description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

It is also to be understood that all base pair and amino acid residue numbers and sizes given
20 for nucleotides and peptides are approximate and used for the purposes of description. Various publications are cited herein that are hereby incorporated by reference in their entireties.

25

30

35

WHAT IS CLAIMED IS:

    1.   A purified and isolated nucleic acid molecule comprising a DNA nucleotide sequence that

       (a)   hybridizes under highly or moderately stringent hybridization conditions to the sequence depicted in Figure 9 (SEQ. ID NO:1); and

       (b)   which is capable of directing the replication and assembly into AAV various, and/or the integration into the host genome, of recombinant DNA containing said nucleic acid molecule.

    2.   A purified and isolated nucleic acid molecule comprising a 165 base pair inverted terminal repeat sequence as depicted in Figure 9 (SEQ ID NO: 1).

    3.   A recombinant DNA vector comprising a DNA nucleotide sequence encoding a protein of interest and a 165 base pair inverted terminal repeat sequence as depicted in Figure 9 (SEQ ID NO: 1).

    4.   A recombinant DNA vector comprising a DNA nucleotide sequence encoding a protein of interest and the nucleic acid molecule of claim 1.

    5.   The recombinant DNA vector according to claim 3 or 4 further comprising a DNA nucleotide sequence that encodes the AAV REP and CAP proteins.

    6.   The recombinant DNA vector according to claim 3 or 4 further comprising a DNA nucleotide sequence recognized by bacterial gamma delta resolvase as depicted in Figure 10 (SEQ ID NO: 2).

- 34 -

7.   The recombinant DNA vector according to claim
5 further comprising a DNA nucleotide sequence
recognized by bacterial gamma delta resolvase as
depicted in Figure 10 (SEQ ID NO: 2).

8.   A method of replicating and encapsidating a
recombinant DNA molecule into an AAV virion,
comprising:

    a)   culturing a eukaryotic cell containing
          (i) a helper virus, (ii) a recombinant
          nucleic acid encoding AAV REP and CAP
          proteins, and (iii) a recombinant
          nucleic acid that contains a DNA
          sequence of interest and a 165 base
          pair inverted terminal repeat sequence
          as depicted in Figure 9 (SEQ ID NO: 1);
          whereby the nucleic acid containing the
          165 base pair inverted terminal repeat
          sequence and the DNA sequence of
          interest is replicated and assembled
          into AAV virions; and
    (b)   collecting virions produced.

9.   A method of replicating and encapsidating a
recombinant DNA molecule into an AAV particle,
comprising:

    a)   culturing a eukaryotic cell containing
          (i) a helper virus, (ii) a recombinant
          nucleic acid encoding AAV REP and CAP
          proteins, and (iii) a recombinant
          nucleic acid that contains a DNA
          sequence of interest and the nucleic
          acid molecule of claim 1; whereby the
          recombinant nucleic acid containing the
          DNA sequence of interest and the
          nucleic acid molecule of claim 1 is

replicated and assembled into AAV
virions; and
(b)   collecting virions produced.

10.   The method according to claim 8 or 9 in
which the helper virus is an adenovirus.

11.   The method according to claim 8 or 9 in
which the helper virus is a herpes simplex virus.

12.   The method according to claim 8 or 9 in
which the recombinant nucleic acid encoding the AAV
REP and CAP proteins is a plasmid vector comprising:
        (a)   promotors which control the expression
               of the AAV REP and CAP RNA;
        (b)   translation initiation signals for the
               REP and CAP mRNA;
        (c)   DNA sequences encoding the REP and CAP
               proteins; and
        (d)   transcriptional termination signals.

13.   The method according to claim 8 in which the
recombinant nucleic acid containing the DNA sequence
of interest and the 165 base pair terminal repeat
sequence is a plasmid vector comprising:
        (a)   a promoter;
        (b)   a DNA sequence of interest transcribed
               under the control of the promoter; and
        (c)   a 165 base pair inverted terminal
               repeat sequence as depicted in Figure 9
               (SEQ ID NO: 1).

14.   The method according to claim 8 in which the
recombinant nucleic acid containing the DNA sequence
of interest and the 165 base pair terminal repeat
sequence is a plasmid vector comprising:
        (a)   a promoter;

- 36 -

       (b)   a DNA sequence of interest transcribed under the control of the promoter; and

       (c)   the nucleic acid molecule of claim 1.

   15.   A method of replicating and encapsidating a recombinant DNA molecule into a viral particle comprising:

       (a)   propagating a recombinant nucleic acid comprising (i) a recombinant viral vector sequence comprising a gene of interest; (ii) viral genes providing helper functions; and (iii) two bacterial gamma delta resolvase recognition sequences as depicted in Figure 10 (SEQ ID NO: 2) flanking the viral vector sequence;

       (b)   treating the propagated nucleic acid with bacterial gamma delta resolvase; and

       (c)   culturing a eukaryotic cell containing a helper virus and said resolvase treated recombinant nucleic acid; whereby the resolved recombinant DNA is replicated and encapsidated into a viral particle.

   16.   A method of replicating and encapsidating a recombinant DNA molecule into an AAV particle comprising:

       (a)   treating a recombinant nucleic acid comprising a 165 base pair terminal repeat sequence as depicted in Figure 9 (SEQ ID NO: 1) and a bacterial gamma delta resolvase recognition sequence as depicted in Figure 10 (SEQ ID NO: 2), with bacterial gamma resolvase enzyme; and

- 37 -

(b) culturing a eukaryotic cell containing
a helper virus and said resolvase
treated recombinant nucleic acid;
whereby the resolved recombinant
nucleic acid is replicated and
encapsidated into an AAV particle.

17. The method according to claim 16 in which
the helper virus is an adenovirus.

18. The method according to claim 16 in which
the helper virus is a herpes simplex virus.

19. The method according to claim 16 in which
the recombinant nucleic acid containing the bacterial
gamma delta resolvase recognition sequence is a
plasmid vector comprising:
(a) a bacterial gamma delta resolvase
recognition sequence;
(b) a promoter;
(c) a DNA sequence of interest transcribed
under the control of a promoter; and
(d) a 165 base pair inverted terminal
repeat sequence as depicted in Figure 9
(SEQ ID NO: 1).

20. The method according to claim 16 in which
the recombinant nucleic acid containing the bacterial
gamma delta resolvase recognition sequence is a
plasmid vector comprising:
(a) a bacterial gamma delta resolvase
recognition sequence;
(b) a promoter;
(c) a DNA sequence of interest transcribed
under the control of a promoter;

- 38 -

        (d)   a 165 base pair inverted terminal
              repeat sequence as depicted in Figure 9
              (SEQ ID NO: 1); and
        (e)   a DNA nucleotide sequence that encodes
              the AAV REP and CAP proteins.

5   21.   A method of transferring genetic information
   into a eukaryotic cell comprising transfection of said
   eukaryotic cell with a recombinant DNA molecule
   containing the nucleic acid molecule of claim 1 or 2.

10   22.   A method of transferring genetic information
   into a host comprising:
        (a). encapsulating a recombinant DNA
              molecule containing the nucleic acid
15   molecule of claim 1 or 2 into a
              liposome; and
        (b)   administering the liposome preparation
              to said host.

20   23.   The method claim 22 in which a nucleic acid
   molecule encoding the AAV REP protein is co-
   encapsulated with the recombinant DNA molecule.

        24.   The method of claim 22 in which AAV REP
   protein is co-encapsulated with the recombinant DNA.
25

        25.   A stock of recombinant adeno-associated
   virus produced according to the method of claim 8
   or 9.

30   26.   A host cell which produces stocks of
   recombinant adeno-associated viruses and produced by a
   method comprising:
        (a)   culturing said host cell containing (i)
              a helper virus, (ii) a recombinant
35   nucleic acid encoding AAV CAP and REP

- 39 -

proteins, and (iii) a recombinant
nucleic acid that contains a DNA
sequence of interest and a 165 base
pair inverted terminal repeat sequence
as depicted in Figure 9 (SEQ ID NO: 1);
5   whereby the nucleic acid containing the
165 base pair inverted terminal repeat
sequence and the DNA sequence of
interest is replicated and assembled
into AAV virions; and
10   (b)   collecting virions produced.

    27.   A eukaryotic cell comprising a recombinant
DNA molecule containing the nucleic acid molecule of
claim 1 or 2.

15

20

25

30

35

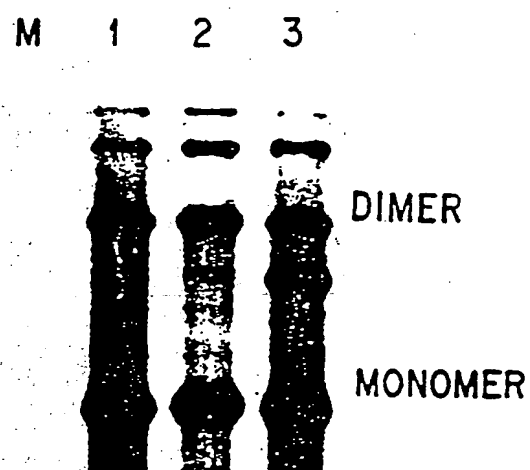FIG.1A

FIG.1B

FIG.1C

FIG.2

FIG.3A   FIG.3B

FIG. 4A

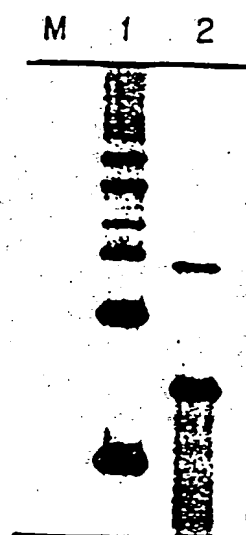DIMER

MONOMER

FIG. 4B

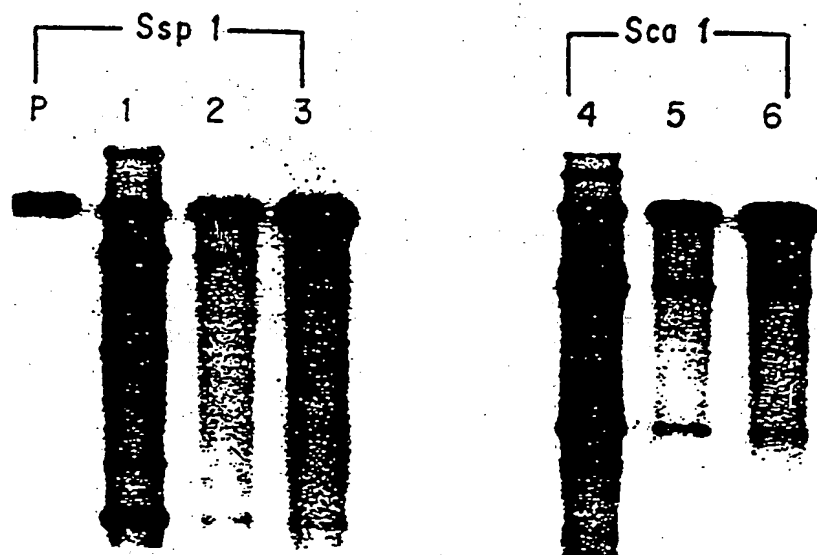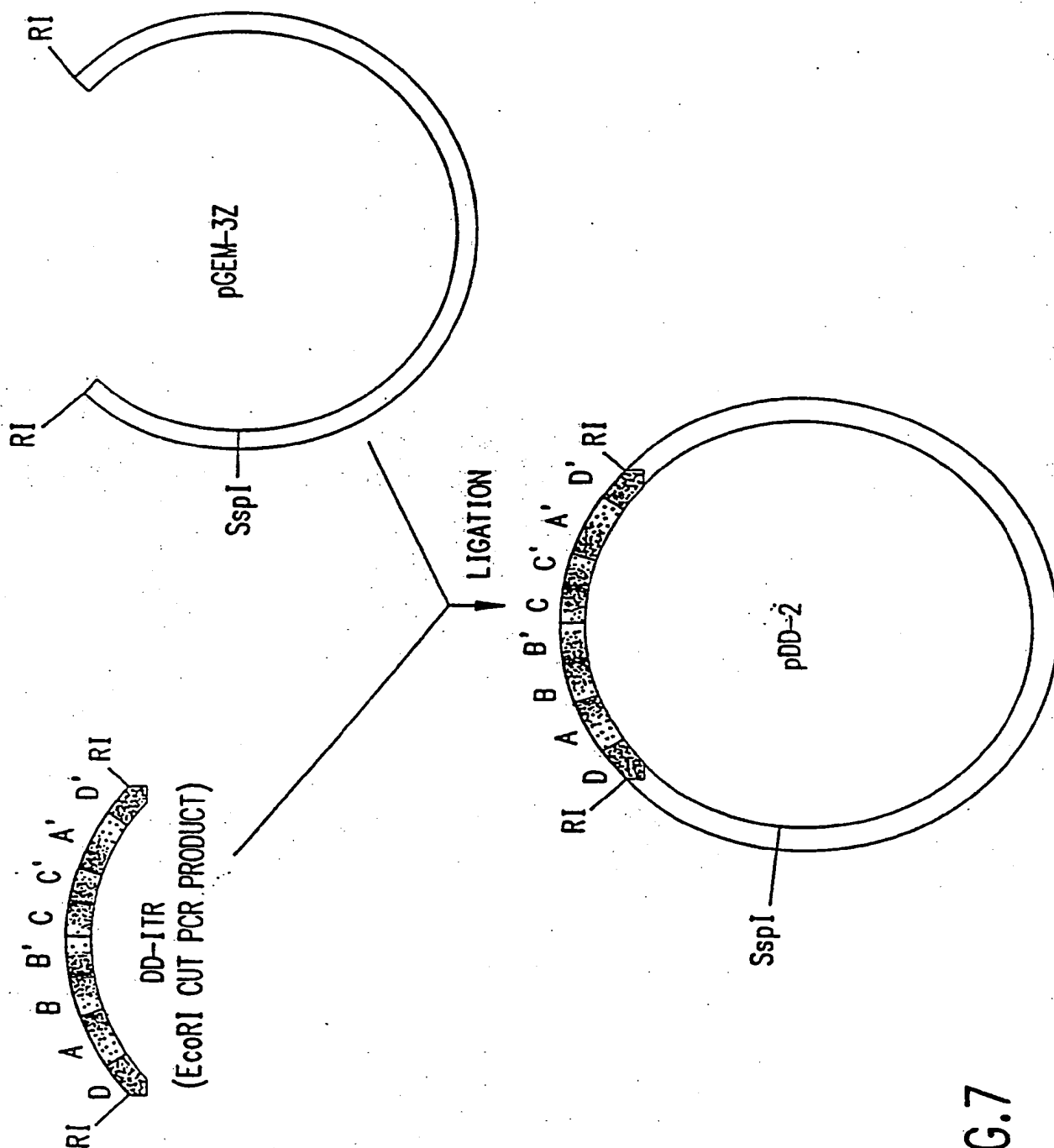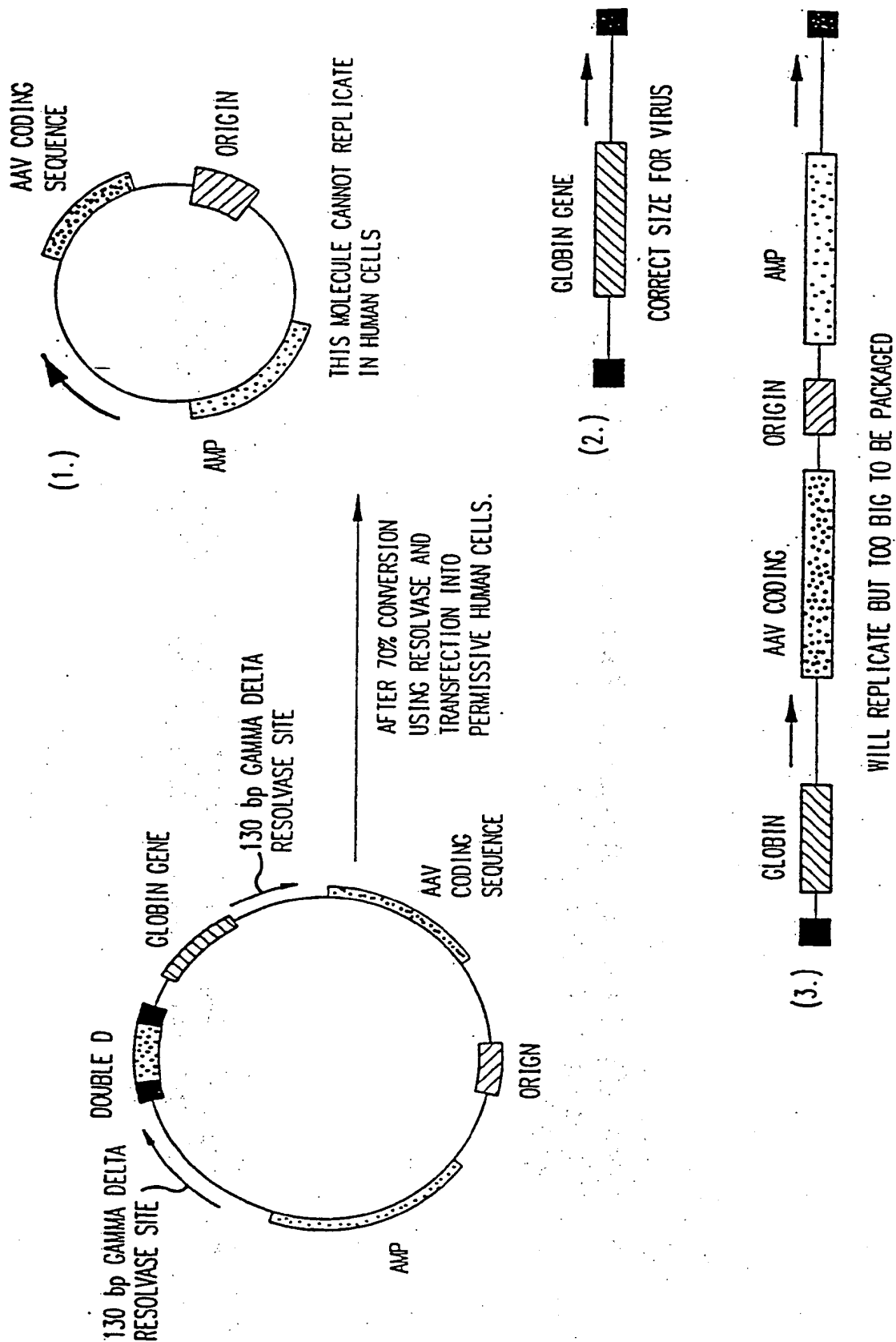FIG.5A   FIG.5B

FIG.6

FIG.7

FIG.8

5 – AGGAACCCCT AGTGATGGAG TTGGCCACTC CCTCTCTGCG CGCTCGGCTCG CTCACTGAGG   60
CCGGGCGACC AAAGGTCGCC CGACGCCCGG GCTTTGCCCG GGCGGCCTCA GTGAGCGAGC   120
GAGCGCGCAG AGAGGGAGTG GCCAACTCCA TCACTAGGGG TTCCT – 3'   165

FIG.9

5'– CCCTGTATCC TAAATCAAAT ATCGGACAAG CAGTGTCTGT TATAACAAAA AATCGATTTA   60
ATAGACACAC CAACAGCATG GTTTTTATGT GTGCGATAAT TTATAATATT TCGGACAGGG – 3'   120
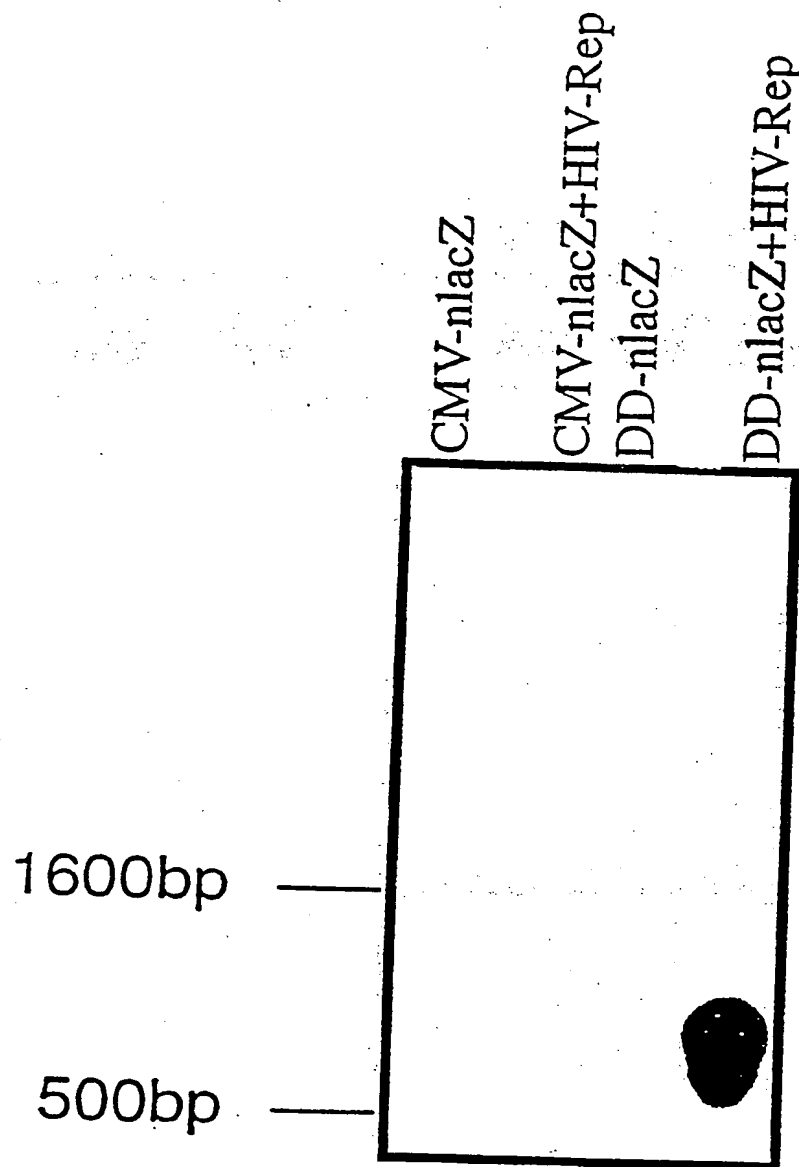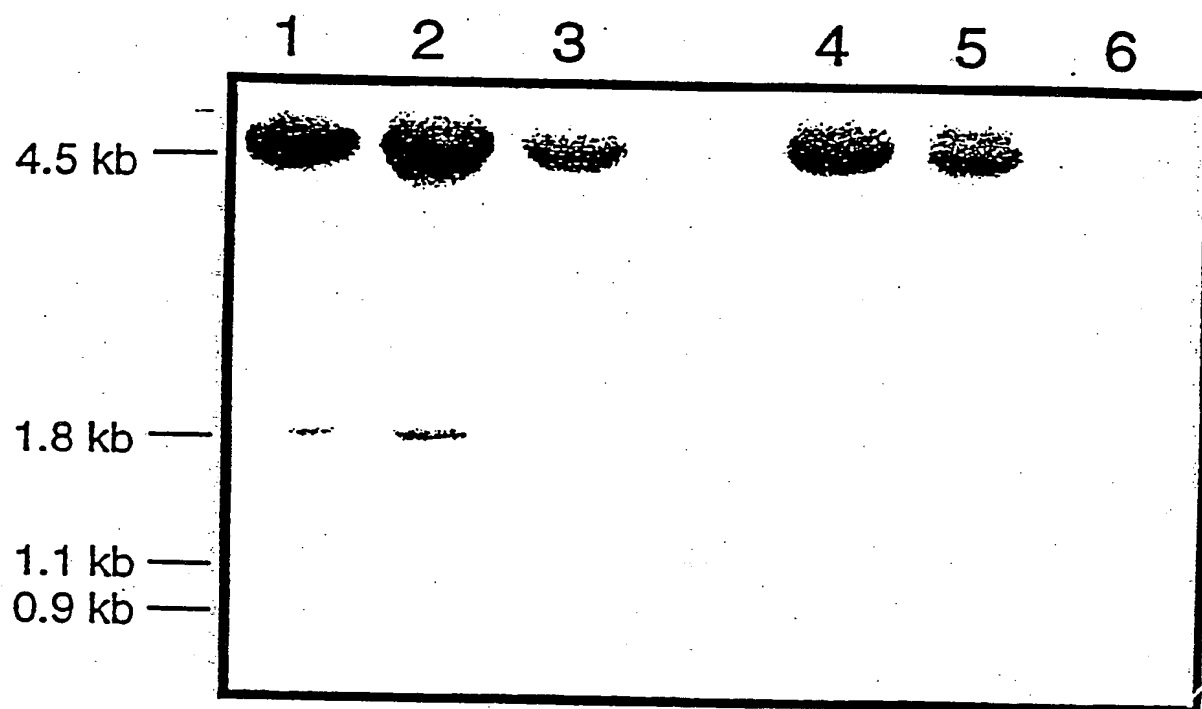
FIG.10

Figure 11

Figure 12

INTERNATIONAL SEARCH REPORT

A.   CLASSIFICATION OF SUBJECT MATTER
IPC(6)   :A61K 48/00; C12N 7/01, 15/10, 15/86; C07H 21/04
US CL   :435/69.1,172.3, 320.1; 536/23.1, 24.1; 424/93.1, 93.6
According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/69.1,172.3, 320.1; 536/23.1, 24.1; 424/93.1, 93.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis, Medline, Biotech
Search Terms: adeno-associated virus, viral vector, gamma delta resolvase, ITR

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,139,941 A (MUZYCZKA et al.) 18 August 1992, Figures 1-2 and columns 6-7.	1-27
A	KOTIN, Prospects for the Use of Adeno-Associated Virus as a Vector for Human Gene Therapy. Human Gene Therapy. 1994, Vol. 5, pages 793-801, especially pages 798-799 and Figure 2.	1-27
A	SAMULSKI et al. Cloning of adeno-associated virus into pBR322: Rescue of intact virus from the recombinant plasmid in human cells. Proceedings of the National Academy of Sciences. March 1982, Vol. 79, pages 2077-2081, especially page 2079.	1-27

☐   Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 AUGUST 1996	04 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID GUZO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

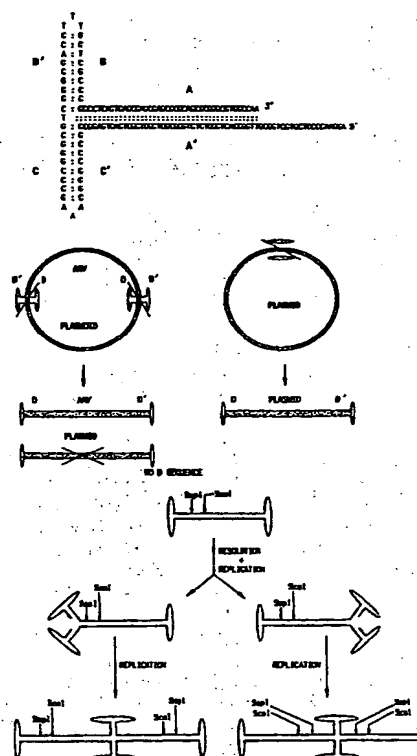INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00, C12N 7/01, 15/10, 15/86, C07H 21/04	A1	(11) International Publication Number: **WO 96/36364** (43) International Publication Date: 21 November 1996 (21.11.96)

(21) International Application Number: PCT/US96/06786

(22) International Filing Date: 14 May 1996 (14.05.96)

(30) Priority Data:
440,738 15 May 1995 (15.05.95) US

(71)(72) Applicants and Inventors: SAMULSKI, Richard, J. [US/US]; 102 Darlin Circle, Chapel Hill, NC 27514 (US). XIAO, Xiao [CN/US]; 210 Riverbirch Lane, Chapel Hill, NC 27514 (US).

(74) Agents: CORUZZI, Laura, A. et al.; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: RECOMBINANT VIRAL VECTOR SYSTEM

(57) Abstract

The present invention relates to a system for replication and encapsidation of recombinant DNA fragments into virus particles comprised of adeno-associated viral (AAV) capsid proteins; said system uses a 165 basepair fragment of DNA which contains AAV inverted terminal repeat sequences and which is used to engineer expression vectors useful for gene therapy. The invention provides a means of obtaining recombinant viral stocks that may be used to treat patients suffering from genetic diseases.

RECOMBINANT VIRAL VECTOR SYSTEM

1. INTRODUCTION

The present invention relates to novel recombinant expression vectors that can be used to introduce and/or express a heterologous gene of interest into targeted host cells. The recombinant expression vectors of the invention contain all the necessary information required *in cis* for site specific integration and/or for replication and encapsidation of the recombinant DNA fragments into virus particles comprised of adenovirus associated viral (AAV) capsid proteins. The novel recombinant expression vectors of the invention provide a means of transferring genetic information, or alternatively, for obtaining recombinant viral stocks that may be used to treat patients suffering from genetic diseases.

2. BACKGROUND OF THE INVENTION

At the present time, treatments for most genetic diseases do little to alleviate the symptoms associated with the genetic disease and considerable effort is currently underway to develop new, safe and effective methods of treatment. Recent progress in the areas of molecular biology and genetic engineering have lead to the isolation and characterization of genes associated with genetic diseases. This in turn has lead to the development of the concept of gene therapy *i.e.*, the replacement or supplement of defective genetic information with normal functional genes, and its potential use for treatment of genetic disorders.

The most well studied models for gene therapy involve gene transfer using recombinant pathogenic viruses to express new genetic information

in order to correct disease phenotypes. Until recently, the most widely researched viral vectors for use in gene therapy were the retroviruses (Miller, A.D., 1990, Human Gene Ther. 1:5-14). One problem associated with retroviral use is the random

5   integration of retroviruses into the host genome which can lead to insertional mutagenesis. In addition, the long terminal repeats (LTR) structures located at the ends of the retroviral genome contain promoter/ enhancer activity that may lead to activation of

10 genetic loci located adjacent to the integrated viral DNA. For example, integration of retroviral DNA adjacent to a proto-oncogene may lead to inadvertent activation of proto-oncogene expression which may, in turn, lead to transformation and tumorigenesis. This

15 is illustrated, for example, by recent evidence indicating that retrovirus vectors in non-human primates results in T cell lymphomas. More recent efforts in the field of gene therapy have focussed on the development of viral vectors lacking the

20 deleterious characteristics of the retroviruses.

        In addition to the retroviruses, the adeno associated viruses have also been studied as an alternative system for delivery of stable genetic information into the cell. The AAV genome is composed

25 of a linear single stranded DNA molecule of 4680 nucleotides which contains major open reading frames coding for the *Rep* (replication) and *Cap* (capsid) proteins. Flanking the AAV coding regions are the two 145 nucleotide inverted terminal (ITR) repeat

30 sequences that contain palindromic sequences that can fold over to form hairpin structures which function as primers during initiation of DNA replication (FIG. 1). In addition, the ITR sequences are needed *in cis*, for viral integration, rescue from the host genome and

35 encapsidation of viral genomic DNA, into mature

- 3 -

virions (Muzyczka, N. 1992, Current Topics in
Microbiology & Immunology. 158, 97-129).

AAV can assume one of two pathways upon
infection into the host cell. In the presence of
helper virus, AAV will enter the lytic cycle whereby
5   the viral genome is transcribed, replicated, and
encapsidated into newly formed viral particles. In the
absence of helper virus function, the AAV genome
integrates as a provirus into of the host cell genome
through recombination between the AAV termini and host
10   cell sequences (Cheung, A. et al., 1980, J. Virol.
33:739-748; Berns, K.I. et al., 1982, in Virus
Persistence, eds. Mahey, B.W.J., et al. (Cambridge
Univ. Press, Cambridge), pp. 249-265).

Characterization of the proviral integration
15   site and analysis of flanking cellular sequences
indicates that AAV viral DNA integrates specifically
into the long arm of human chromosome 19 (Kotin, R.M.
et al., 1990, Proc. Natl. Acad. Sci. USA 87:2211-2215;
Samulski, R.J. et al., 1991, EMBO J. 10:3941-3950).
20   This particular feature of AAV reduces the likelihood
of insertional mutagenesis resulting from random
integration of viral vector DNA into the coding region
of a host gene. Furthermore, in contrast to the
retroviral LTR sequences, the AAV ITR (inverted
25   terminal repeat) sequences appear to be devoid of
transcriptional regulatory elements, reducing the risk
of insertional activation of proto oncogenes.

Recent work with AAV has been facilitated by
the discovery that AAV sequences cloned into
30   prokaryotic vectors are infectious (Samulski, et al.
1982, Proc. Natl. Acad. Sci. U.S.A. 79:2077-2081).
For example, when a plasmid containing an intact AAV
genome is transfected into cells in the presence of
helper virus, AAV can be rescued out from the plasmid
35   vector and enter the lytic pathway leading to
production of mature virions. In the absence of

- 4 -

helper virus the recombinant AAV vector will integrate
into the host cell genome and remain as a provirus
until the cell subsequently becomes infected with a
helper virus.

5   One problem associated with the use of
vectors containing an intact AAV genome is the size
limitations imposed on the vectors by the packaging of
DNA fragments into mature viral particles. Prior to
the present invention it was unknown what viral
sequences were required for replication, and
10   encapsidation of the DNA into viral particles, or
alternatively, for integration of vector DNA into the
host genome. The present invention defines a novel
165 bp sequences derived from AAV that can function to
direct the replication and encapsidation of DNA into
15   viral particles and/or the integration of vector DNA
into the targeted host genome.

3.   SUMMARY OF THE INVENTION

          The present invention relates to the
in vitro synthesis of a novel 165 basepair fragment of
20   DNA which contains AAV ITR sequences and which can be
synthesized *in vitro* and used to engineer expression
vectors and/or vectors useful for genetic therapy.
This 165 bp DNA sequence, herein referred to as the
"double-D sequence," is in a novel configuration not
25   found to exist in wild type AAV.

          The invention is based, in part, on the
ability of the double-D sequence to provide sufficient
information, *in cis*, for converting a circular duplex
DNA molecule into a linear replicating molecule with
30   covalently closed ends. The replicating DNA molecule
may be either encapsidated into mature AAV virions, or
integrated into the host genome. A particularly
significant feature of the circular duplex DNA
molecule is that the process of conversion into a
35   linear molecule, and the replication and integration

into the host genome is completely effected through
the double-D sequences, thus ensuring that the
heterologous gene sequences of interest remain intact.

The discovery that the majority of the AAV
genome is not needed, in cis, for replication,
packaging and/or integration allows one to insert
larger fragments of DNA into the recombinant vectors.
In addition, the discovery that the 165 double-D
sequence is sufficient for targeting vector DNA to
chromosome 19 allows the insertion of any sized DNA
fragment into the recombinant vectors, thereby
removing any size restraints.

Nucleotide sequences which may be used in
accordance with the invention include derivatives and
analogs of the double-D sequences disclosed herein
that are functionally equivalent in that they retain
their ability to provide information, in cis, for
replication encapsidation, integration and rescue of
recombinant DNA. In particular, double-D derivatives
may contain additions, substitutions or deletions of
the double-D nucleotide sequences while retaining
their biological function.

The invention provides an in vivo system for
replication and packaging of recombinant DNA into
mature virions. Viral stocks containing the
recombinant DNA encapsidated into mature virions, may
be obtained by transfection of expression vectors into
a host cell expressing helper function. The resulting
recombinant viral stocks afford a convenient and
efficient means for transfer of genetic information
into any cell or tissue of choice.

Alternatively, the double-D constructs may
be delivered into host cells using a variety of DNA
transfer methods such as electroporation, DEAE-
dextran, DNA gun, liposomes etc. One particular
advantage associated with these delivery methods is

- 6 -

the absence of size restraints on the size of the
recombinant vectors.

The transfer of genetic information into
host cells, using either viral stocks or transfection
of recombinant constructs, may have applications in
gene therapy wherein the desired goal is correction of
a given genetic abnormality through expression of the
normal complement of the defective gene.

The invention also relates to *in vitro*
treatment of recombinant viral vectors and/or vectors
useful for genetic therapy with bacterial gamma delta
resolvase prior to transfection. Genetic engineering
of resolvase recognition sequences into recombinant
vectors creates the option of removing bacterial
plasmid sequences which would normally be included as
part of the linear, replicated, and encapsidated DNA
molecule. Removal of these bacterial sequences, prior
to replication and encapsidation, allows the maximum
amount of space for insertion of foreign DNA sequences
of interest into the expression vectors.

The invention is described by way of
examples in which an AAV double-D fragment is
amplified in a polymerase chain reaction (PCR) and
inserted into a plasmid vector. Transfection and site
specific integration of these double-D recombinant
vectors into the host genome indicates that double-D
sequences, in the presence of viral REP protein, are
sufficient to direct site specific integration of
vector DNA. In addition, plasmid vectors containing
the double-D sequences were capable of being
replicated and assembled into viral particles. Thus,
the present invention provides a method for
encapsidating recombinant DNA into viral particles
that may be used for gene therapy.

- 7 -

4.   BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1.   AAV rescue and replication mechanisms.   (A) Inverted terminal repeats (ITR) may fold back through the base-pairing of A,A', B,B',C,C' sequences to form a T-shape structure.   (B) Excision of infectious plasmid at the ITR sites yields two linear DNA fragments : AAV and the plasmid vector. (C) Predicted fragments generated from double D plasmid after rescue from plasmid circular form to linear with covalent closed ends.

FIG. 2.   Rescue Intermediate from circular double-D plasmid.

FIG. 3.   Replication assay for the plasmid containing the double-D inverted terminal repeat. Plasmid pDD-2 was transfected into Ad5 infected 293 cells with or without cotransfection of the helper plasmid pAAV/Ad.   Low molecular weight DNA was extracted 48 hrs. post infection and separated on a 1% agarose gel with or without DpnI digestion.   (A)- Ethidium bromide staining of the gel.   (B) Southern blot with ^{32}P labeled plasmid pGEM 3z probe.

FIG. 4.   Comparison of replication of pDD-2 with psub201 and pSM620.   Plasmid pDD-2 was cotransfected with equal amounts of either pAAV/Ad (lane 1), psub201 (lane 2) or pSM620 (lane 3) into Ad5 infected 293 cells.   Low molecular weight DNA was extracted and digested with DpnI and analyzed on a 1% agarose gel.   (A) Ethidium bromide staining.   (B) Southern blot with an ITR oligonucleotide probe.

FIG. 5.   Replication assay for double-D plasmids with different sizes.   pDD-2 or pDD-neo were cotransfected with helper pAAV/Ad into Ad5 infected 293 cells.   Low molecular weight DNA was extracted and digested with DpnI and analyzed on a 1% agarose gel. (A)Ethidium bromide staining.   (B)Southern blot with ^{32}P labeled plasmid pGEM 3Z probe.

- 8 -

FIG. 6.   Restriction analysis of rescue and replication of pDD-2. Plasmid pDD-2 was transfected into Ad5 infected or uninfected 293 cells with or without the cotransfection of helper plasmid pAAV/Ad. Low-molecular-weight DNA was digested with either SspI or ScaI and separated on a 1% agarose gel. Southern blot was done with a ^{32}P labeled ITR probe.

FIG. 7.   Cloning of PCR-amplified and EcoRI cut double-D into EcoRI site of plasmid pGEM-3'Z.

FIG. 8.   *In-vitro* replication of parental plasmid containing resolvase sequences.

FIG. 9.   Double-D sequence (SEQ ID NO: 1).

FIG. 10.   Bacterial Gamma Delta Resolvase recognition sequence (SEQ ID NO: 2).

FIG. 11.   PCR amplification of DD-cellular junctions. Genomic DNA was isolated from pooled FACS-sorted 293 cells that has been transfected by different combination of CMV-nLacZ, DD-nLacZ and HIV-Rep plasmid. The first round PCR was performed with JUS3A and RI-Left, the second round PCR reaction was carried out with CR2 and RI-left primers. The Southern blot was probed with a ^{32}P labelled BamHI fragment corresponding to a region chromosome 19.

FIG. 12   Southern blot analysis of the integrated plasmids in FACS sorted 293 genomic DNA. Each lane contains 10 μg of DNA digested by ScaI, PstI and KpnI. The DNA was fractionated on 7% gel and the probe was made from DD-nLacZ plasmid. Lane 1 to 6, 293 cells transfected by DD-nLacZ plasmid, DD-nLacZ plasmid+Rep, DD-nLacZ plasmid+HIV-Rep, CMV-nLacZ plasmid, CMV-nLacZ plasmid+REP, CMV-nLacZ plasmid+HIV-Rep.

5.   DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to *in vitro* construction of a novel modified AAV ITR nucleotide sequences, herein referred to as the double-D

sequence. The invention is based, in part, on the
synthesis of a double-D DNA fragment using PCR
technology, and the demonstration that this fragment
provides sufficient information in cis for replication
and encapsidation of recombinant DNA fragments into
5   mature AAV virions, or the specific integration of
recombinant DNA into the host genome. The recombinant
DNA fragments need not encode any viral proteins
thereby providing a safe method for the transfer of
genetic information into the host genome.

10   The invention is based on the discovery that
the cis-acting double-D sequences, in conjunction with
trans-acting AAV REP protein, are sufficient for site
specific integration of recombinant DNA fragments into
chromosome 19 of the host genome. The invention
15   relates to the use of the double-D DNA sequences in
recombinant vectors and the use of these vectors for
gene replacement therapies.

The double-D sequences may be genetically
engineered into vectors designed to introduce and/or
20   express a heterologous gene of interest. For use in
gene therapy, the heterologous gene of interest may
represent the normal or wild-type version of a
defective or mutant gene associated with a given
genetic disease. The recombinant vectors, in addition
25   to containing the coding region for the gene of
interest and the double-D sequences, may contain other
necessary regulatory elements such as promoter/
enhancer elements to drive expression of the gene
product in the host, and translation and
30   polyadenylation signals. The selection of promoter
and enhancer regions will rely on the desired level
and tissue specific expression of the gene of
interest.

The genetically engineered vectors
35   containing the double-D sequences can be transfected
into host cells utilizing any of the techniques

frequently employed by those skilled in the art for
introducing DNA into mammalian cells. For example,
methods including but not limited to electroporation,
DEAE-dextran mediated DNA transfer, DNA guns,
liposomes, direct injection, etc. may be utilized to
transfer recombinant vectors into host cells.
Alternatively, the DNA may be transferred into cells
through conjugation to proteins that are normally
targeted to the inside of a cell. For example, the
DNA may be conjugated to viral proteins that normally
target viral particles into the targeted host cell.

        Host cells may be transfected *in vivo* or
in vitro. For example, cells such as bone marrow
cells, may be removed from the host, transfected with
the recombinant double-D vectors and returned to the
host. Alternatively, recombinant double-D vectors may
be encapsulated into liposomes for *in vivo* transfer of
genetic information into host cells.

        One significant advantage associated with
transfection of double-D vectors into host cells is
the absence of restraints on the size of the
recombinant vectors. This is in contrast to the size
limitations imposed by the packaging of DNA fragments
into mature virus particles. Another advantage is
that there is no risk of generating infectious viral
particles when the treated individual later becomes
infected with helper virus and AAV.

        The recombinant vectors may also be
transfected into a host cell line that provides helper
virus function and supplies *in trans* the *REP* and *CAP*
proteins allowing one to obtain a recombinant virus
stock (Muzyczka, N. 1992, Current Topics in
Microbiology and Immunology 158:97-129). The
resulting virus stock may then be used as a highly
efficient means of delivery and transfer of new
genetic information into targeted cells or tissues of
choice.

- 11 -

5.1. THE DOUBLE-D SEQUENCES

The AAV genome consists of 4680 nucleotides
containing two open reading frames that encode the *REP*
(replication) and *CAP* (capsid) proteins. Located at
both ends of the genome are 145 bp inverted terminal
repeats (ITRs), which are unique in that they not only
basepair with each other, but also individually fold
back on themselves through the basepairing of A, A',
B, B', C, C' sequences to form a T-shaped structure
for DNA replication when single stranded (FIG. 1A).

When a plasmid containing an intact AAV
genome is transfected into helpervirus infected cells,
AAV can be rescued or replicated out from the plasmid
vector and enter the viral lytic cycle leading to
production of mature virions. If the AAV coding
region is deleted and replaced by heterologous DNA
sequences, the recombinant AAV can still complete the
viral lytic cycle provided the ITRs are intact and the
REP and *CAP* proteins, or functional equivalents, are
supplied *in trans*. However, if one of the two ITR
sequences are deleted no viral DNA replication is
observed indicating that both ITRs are required for
AAV viability.

The invention is based, in part, on the
discovery that the following 20 basepair D sequence
(AGGAACCCCTAGTGATGGAG) (SEQ ID NO: 5), present in the
ITR sequence I required for viral replication. This
is initially demonstrated by the inability of viral
mutants with deleted D sequences to replicate their
DNA   Furthermore, during the replication of a
terminal resolution site mutant, natural deletions
were found to occur only towards the A sequence of the
ITR and not towards the D end, suggesting a selection
process for retention of D sequences.

In order to elucidate further the function
of the D sequences, a novel modified terminal repeat
structure was constructed containing a single 145 bp

- 12 -

ITR sequence with an additional 20 bp D' sequence
(FIG. 9) (SEQ ID NO: 1) (See Section 6.13, _infra_).
The resulting 165 bp sequence has not been identified
in any naturally occurring virus. Using AAV DNA as
template and a single primer derived from the D
5   sequence of the AAV ITR sequence plus a 6bp EcoRI
recognition site on the 5' end, a polymerase chain
reaction was performed that resulted in a DNA fragment
comprised of an ITR flanked on either side by D or D'
sequences as well as EcoRI sites. The PCR generated
10 DNA fragment was cleaved by EcoRI and subsequently
cloned into the EcoRI site of pGEM3Z (FIG. 7).

To determine whether the double-D was able
to function in replication, encapsidation, integration
and rescue of recombinant DNA, a recombinant plasmid
containing the double-D structure was transfected into
15 cells . Results from these experiments indicate that
the novel double-D sequence is sufficient to carry out
the functions normally required of two wild type ITRs
during a lytic AAV viral infection.

In addition, cotransfection of recombinant
20 DNA containing double-D sequences and DNA encoding AAV
REP protein indicate that the double-D sequences and
the _REP_ protein are all that is required for site
specific integration into the host genome. (See
Section 6.2.3. and Section 6.2.4., _infra_). Analysis
25 of the viral integration site indicates that
integration takes place through the ITR sequences,
thus ensuring that the vector sequences, i.e. those
sequences containing heterologous sequences of
interest remain intact.
30
In addition to the double-D sequence of FIG.
9 (SEQ. ID NO.: 1), nucleotide sequences capable of
hybridizing to the double-D sequences (SEQ ID NO:1)
under highly or less highly stringent hybridization
conditions are well within the scope of the invention.
35 Highly stringent hybridization conditions may be

- 13 -

defined as hybridization to filter-bound DNA in 0.5 M
NaHPO4, 7% sodium dodecyl sulfate (SDS), 1mM EDTA at
65°C, followed by washing in 0.1 x SSC/0.1% SDS at
68°C (Ausubel F.M. et al., eds, 1989, Current
Protocols in Molecular Biology, Vol. I, Green
Publishing Associates, Inc., and John Wiley & Sons,
Inc., New York at p. 2.10.3). Less highly stringent
conditions, such as moderately stringent conditions,
may be defined as hybridizations carried out as
described above, followed by washing in 0.2x SSC/0.1%
SDS at 42°C (Ausubel et al., 1989, supra).

In an additional embodiment of the
invention, double-D sequences from other adenovirus-
associated viral subtypes which form hairpin
structures, may be used for isolating double-D DNA
fragments. Alternatively, altered nucleotide
sequences which may be used in accordance with the
invention include derivatives and analogs of the
double-D sequence that are functionally equivalent in
that they retain their ability to provide information,
in cis, for replication, encapsidation, integration
and rescue of recombinant DNA. In particular,
double-D derivatives may contain additions,
substitutions or deletions of nucleotide sequences but
still retain biological function.

The present invention includes methods for
identifying additions, substitutions or deletions of
double-D sequences that retain double-D function.
Alterations in the double-D sequences may be generated
using a variety of chemical and enzymatic methods
which are well known to those skilled in the art. For
example, oligonucleotide-directed mutagenesis may be
employed to alter the double-D DNA sequence in a
defined way and/or to introduce restriction sites in
specific regions within the double-D sequence.
Alternatively, deletion mutants may be generated using
DNA nucleases such as Bal 31 or Exo III and S1

- 14 -

nuclease. Progressively larger deletions in the
double-D sequences may be generated by incubating said
DNA with nucleases for increased periods of time.
(See Ausubel, et al., 1989 Current Protocols for
Molecular Biology, for a review of mutagenesis
techniques).

5

The altered double-D sequences may be
evaluated for their ability to provide information,
in cis, for replication, encapsidation, integration
and rescue of recombinant DNA using, for example, any
of the methods described herein (See e.g., Section 6

10

supra). It is well within the scope of the present
invention that any altered double-D sequences that
retain their ability to provide information, in cis,
for replication, encapsidation, integration and rescue
of recombinant DNA may be incorporated into

15

recombinant expression vectors which may then be
employed for transfer of genetic information into host
cells.

A number of advantages are associated with
the use of recombinant vectors containing double-D

20

sequences. One advantage is that the double-D
sequences enable these vectors to convert from
circular duplex molecules into linear replicating
molecules with covalently closed hairpin ends (see
Figure 6). This is believed to be essential as the

25

formation of linear molecules is a major prerequisite
for successful integration of recombinant DNA into the
host genome. The spontaneous formation of linear
molecules, in the absence of any viral proteins,
represents a significant advantage over the use of

30

other AAV based vectors which require linearization
prior to introduction by transfection into host cells.

Additionally, the formation of linear
replicating molecules and the integration of the
replicated molecules via the double-D sequences

35

- 15 -

ensures that the DNA sequences ensures that the DNA
requires encoding the gene of interest remain intact.

5.2. CONSTRUCTION OF RECOMBINANT VECTORS COMPRISED OF DOUBLE-D SEQUENCES AND HETEROLOGOUS LINKED SEQUENCES

5   The double-D sequences (SEQ ID NO: 1) of the
invention provide all the necessary information for
directing the replication and encapsidation of
recombinant DNA into mature virions. A DNA fragment
containing a double-D nucleotide sequence may be
10   obtained by any number of methods commonly used in the
art. In a specific embodiment, described herein, a
polymerase chain reaction (PCR) was used to obtain a
double-D DNA fragment using AAV DNA as template and a
primer derived from the D sequence of the AAV ITR.
15   The rationale for this approach is based on the
expected secondary structure of the natural ITR
sequence.

            In the first round of the PCR reaction, the
AAV viral ITR forms a hairpin structure and self-
20   primes the elongation process to produce a long
T-shaped hairpin structure containing D and D' on the
stem. Upon denaturation, this DNA serves as template
for a single-primed PCR reaction. Alternative methods
for isolating a double-D DNA fragment, include but are
25   not limited to chemically synthesizing the DNA
sequence.

            Standard recombinant DNA methods may be used
for insertion of novel double-D sequences into
recombinant vectors which may include, for example,
30   plasmid or cosmid vectors. The methods include *in
vitro* recombinant DNA techniques, synthetic techniques
and *in vivo* recombination/genetic recombination. For
example, the double-D DNA sequence may be amplified in
a PCR reaction using oligonucleotide primers that add
35   an appropriate restriction endonuclease recognition

- 16 -

site onto each end of the amplified DNA fragment (See
Section 6.1.3). Alternatively, any restriction site
desired may be produced by ligating nucleotide
sequences (linkers), comprising specific chemically
synthesized oligonucleotides encoding restriction

5   endonuclease recognition sequences, onto the termini
of the amplified double-D fragment into an expression
vector having complementary cohesive termini.

            A variety of host recombinant vector systems
may be utilized equally well by those skilled in the

10   art. The recombinant vectors could contain bacterial
plasmid sequences necessary for replication in *E.coli*
or the *cis* acting double-D sequence could be ligated
directly to the gene of interest. In addition,
plasmids will contain DNA sequences coding for a

15   heterologous gene of interest, inserted between the
appropriate transcriptional/translational control
sequences and polyadenylation signals. A variety of
promoter/enhancer elements may be used depending on
the level and tissue specific expression desired.

20   Promoters produced by recombinant DNA or synthetic
techniques may be used to provide for transcription of
the inserted gene of interest. Specific initiation
signals are also required for efficient translation
of inserted protein coding sequences. These sequences

25   include the ATG initiation codon and adjacent
sequences. In addition, polyadenlylation signals may
be included to increase the stability of transcribed
mRNA.

            One potential drawback of the AAV viral
vector system is the size limitation imposed by the

30   inability of DNA fragments larger than 5 Kb to be
packaged into mature virus particles. In any given
expression vector, 2-3 Kb of DNA sequence derives from
bacterial plasmid sequences which are required for
propagation of plasmid in *E. coli*. These DNA

35   sequences include origin of replication (*ori*)

sequences and genes that confer resistance to
antibiotics such as ampicillin and tetracycline. In
effect, this leaves only 2 Kb of space for insertion
of heterologous genes of interest.

The following particular, non-limiting
embodiment of the invention addresses this size
limitation problem. To increase the amount of space
available for cloning of sequences of interest, a
bacterial recombination system (gamma delta) may be
used to resolve plasmids in such a manner that the
majority of the bacterial plasmid DNA sequences will
be recombined out of any given recombinant plasmid
construct *in vitro*, thereby allowing for the maximum
amount of space for insertion of foreign genes. The
gamma delta resolvase sequences may be used in a
variety of viral vector systems, not limited to AAV
systems, as a general method for increasing the space
available for insertion of foreign genes.

The parental double-D expression vector
plasmid may be engineered to contain, in addition to
the double-D sequence, two copies of the gamma delta
resolution site (FIG. 10) (SEQ ID NO: 2) (120
basepairs) in the correct orientation so as to promote
recombination when resolved *in vitro* with gamma delta
resolvase enzyme. In the presence of gamma delta
resolvase, the recombinant plasmid should be converted
into two circular DNA molecules (FIG. 8). One plasmid
molecule should contain the primary bacterial plasmid
sequences along with a copy of the gamma delta
resolution site. The other resolved plasmid molecule
would be expected to contain the double-D *cis* acting
sequences, one copy of a gamma delta resolution site
and the coding region for the gene of interest. It is
this plasmid molecule which will be converted, in the
presence of helper virus and the viral *REP* and *CAP*
proteins, into a linear replicating molecule which
will then be encapsidated into mature viral particles.

- 18 -

Currently, a second plasmid is required to supply the *CAP* and *REP* proteins which are needed to convert circular plasmid molecules into replicating linear DNA fragments. In a further particular embodiment of the invention, plasmids containing the

5   gamma delta resolution sites may be engineered to also contain the coding regions for the required viral *REP* and *CAP* functions. Normally the *REP* and *CAP* coding sequences would be excluded from the expression vector because they further limit the size of your insert.

10 Using the *in vitro* recombination system, these coding regions may be included in the plasmid construct, since they will be recombined out during resolution through the two gamma delta resolution sites by the resolvase enzyme (FIG. 8). The products are two concatenated DNA molecules.

15

Because the *in vitro* gamma delta reaction is a linear reaction, the amount of resolved molecules may be controlled in the *in vitro* resolving reaction to generate desired ratios of parental plasmid to resolved circles. The mixture of plasmids can then be

20 transfected into the host cell. This introduces one circular molecule containing the AAV *REP* and *CAP* genes required *in trans* for a productive replication, and the other circular molecule contains the double-D sequences and the gene of interest. The double-D

25 circular molecule may be replicated into a linear DNA molecule which may subsequently be encapsidated into viral particles by the *trans* factors introduced on the REP/CAP circular plasmid.

The present invention further provides for

30 the analogous use of the above-described gamma delta resolvase system to first propagate plasmids that comprise (i) a recombinant viral vector sequence comprising a gene of interest, (ii) viral genes providing helper functions, and (iii) two gamma delta

35 resolvase recognition sequences flanking the viral

- 19 -

vector sequence (SEQ ID NO: 2) and, second, to
separate recombinant viral vector sequences comprising
the gene of interest from the remaining plasmid
sequence using resolvase enzyme. According to these
methods, the virus from which the vector and helper

5   functions are derived may be any suitable virus
including but not limited to AAV, a retrovirus,
adenovirus, or herpes virus. In preferred embodiments
the viral vector portion of the plasmid comprises the
double-D sequence or, alternatively, both AAV ITR's.

10   In general, the viral vector protein comprises
sequences necessary for encapsidation and
transcription of the gene of interest.

5.3. PRODUCTION OF RECOMBINANT VIRUS STOCKS

15   The invention relates to a method for
replicating and encapsidating a recombinant DNA
molecule into an AAV particle which comprises
culturing a eukaryotic cell containing helper virus,
recombinant DNA encoding AAV *REP* and *CAP* proteins, and

20   a recombinant nucleic acid containing a DNA sequence
of interest and the 165 base pair double-D sequence.

             To generate recombinant viral stocks, the
double-D recombinant expression vector plasmid may be
transfected into a host cell line that is capable of

25   providing helper virus function, and supplying *in
trans* AAV *REP* and *CAP* proteins. The *REP* and *CAP*
proteins are required for replication and
encapsidation of the linear recombinant DNA into
mature viral particles.

30   The *REP* and *CAP* proteins may be supplied *in
trans* by transfection of the host cell line with a
recombinant plasmid that is capable of coding for each
of the proteins. DNA transfections may be carried out
using methods well known to those skilled in the art.

35   These may include DNA transfection by lipofection,
electroporation or calcium phosphate precipitation

- 20 -

(Ausubel, et al., 1989, in Current Protocols for
Molecular Biology, Green Publishing Associates, Inc.
and John Wiley & Sons, Inc., New York). The plasmid
is transfected into the host cell line with the
intention of either transiently or stably expressing

5   the REP and CAP proteins. In a specific embodiment,
described in Section 6.1., the plasmid pAAV/AD
containing the AAV coding regions, was transfected
into a host cell for the purpose of expressing the REP
and CAP proteins.

10   In another embodiment, the double-D
expression vector may be engineered to directly
express the REP and CAP proteins. In this case, it is
also important to include the gamma delta resolvase
sequences in the plasmid vector, so that the REP and

15 CAP coding regions may be recombined out during an in
vitro resolvase reaction so as not to impose a size
limitation on the insert of foreign DNA.

        In addition to expressing the viral REP and
CAP proteins, the host cell lines must be able to

20 provide helper virus function. Both adenovirus and
herpes simplex virus may serve as helper viruses for
replication of DNA fragments containing the double-D
sequences. Any host cell permissive for infection by
either of these two viruses or any virus that acts as

25 a helper virus for AAV, may be used in the practice of
the invention. The multiplicity of infection (MOI)
and the duration of the infection time will depend on
the type of virus used and the cell line employed.

        In a specific embodiment, described herein,

30 293 cells which had previously been transfected with a
recombinant double-D expression vector, were infected
with Ad5 at a MOI of 10. Forty-eight hours later the
cells were frozen and thawed three times, and
incubated for one hour at 56°C to inactivate the

35 adenovirus. The resulting cell lysate contains

recombinant viral particles that may be used to infect
cells or tissue of choice.

Alternatively, the recombinant double-D DNA
vectors may be grown up and purified for use in DNA
transfections, using any of the methods well known to
5   those skilled in the art. (See *Sambrook et al.*, 1989,
Molecular Cloning: A Laboratory Manual, Cold Spring
Harbor Laboratory Press, Cold Spring Harbor, N.Y., for
description of methods that may be used to propagate
recombinant vector DNA.)

10

5.4.   USES OF RECOMBINANT VECTORS

The double-D expression vectors, containing
a heterologous gene of interest and described herein,
may be useful for therapeutic treatment of genetic
15   disorders. The gene of interest may be the wild type
complement of any mutated or defective gene and may be
inserted into the double-D recombinant vectors so that
its expression is controlled by its natural promoter
(*e.g.*, so that expression is regulated normally) or by
20   a heterologous promoter.

The double-D expression vectors may be
transfected into host cells using any of the
techniques routinely used by the skilled artisan for
efficient transfer of nucleic acids into host cells.
25   Host cells may be transfected *in vivo* or *in vitro*.
For example, cells may be removed from the host and
transfected with recombinant vectors. The transfected
cells may be assayed for proper integration of the
recombinant DNA into chromosome 19 of the host. The
30   cells may also be tested to determine whether the gene
product of interest is properly expressed. Following
the verification of proper integration and expression
of the recombinant DNA, the transfected cells may be
implanted or returned into the host.

Alternatively, transfer of recombinant DNA
35   may take place *in vivo*. In a preferred embodiment of

- 22 -

the invention the recombinant double-D vectors may be
encapsulated into liposomes for delivery into host
cells. Liposomes are spherical lipid bilayers with
aqueous interiors. All molecules that are present in
an aqueous solution at the time of liposome formation
(in this case, recombinant vectors and/or REP protein)
are incorporated into this aqueous interior. The
liposomal contents are both protected from the
external microenvironment and, because liposomes fuse
with cell membranes, are efficiently delivered into
the cell cytoplasm. The double-D vectors of the
invention, may be co-encapsulated into liposomes with
viral *REP* protein, RNA molecules encoding *REP* protein
or viral DNA sequences encoding the *REP* protein, to
provide for site specific integration of viral vector
sequences into the host chromosome.

Additionally, recombinant viral stock may be
generated by transfection of the double-D plasmid into
a suitable host cell line that first allows for
conversion of a circular duplex molecule into a linear
DNA molecule covalently closed at both ends. This
then permits replication and encapsidation of
recombinant DNA into mature viral particles. The
resulting viral stocks may then be used to infect
tissue or cells affected by the genetic defect.

6. EXAMPLE: A NOVEL 165 BASE PAIR TERMINAL
    REPEAT IS THE ONLY *cis*-ELEMENT REQUIRED
    FOR ADENO-ASSOCIATED VIRUS LIFE CYCLE

The subsection below describes the synthesis
and functional characterization of the double-D
sequence.

6.1. MATERIALS AND METHODS
6.1.1. DNA TRANSFECTION
Human cell line 293 was maintained in DMEM
(Dulbecco modified Eagle medium, GIBCO) WITH 10% FCS
(fetal calf serum, HyClone). Transfection of plasmid

- 23 -

DNA was done by lipofection (BRL) method as described
by the manufacturer. Briefly, cells in a 6-cm dish
were washed twice with DMEM and infected with
Adenovirus 5 at 10 moi (multiplicity of infection) in
1ml Opti-MEM (GIBCO) for 1 hr. Then 5 ug plasmid DNA
was incubated with 50 ul of lipofectin (BRL) at room
temperature for 10 min, mixed with 2 ml of Opti-MEM
and added to the Adenovirus infected cells. After
incubation for 12 hrs., the cells were fed with 3 ml
of DMEM containing 4% FCS and incubated for an
additional 36 hours.

6.1.2.   SOUTHERN HYBRIDIZATION

Low molecular weight DNA from transfected
cells was extracted as described by Hirt (Hirt,
B.1967, J. Mol. Biol. 26:365-369). The DNA was
digested by restriction enzymes (New England BioLab),
separated on an agarose gel, then transferred onto the
Genescreen plus Nylon membrane (DuPont).
Hybridization with ^{32}P labeled plasmid DNA was carried
out as recommended by the manufacturer. Hybridization
with r- ^{32}P-ATP end-labeled ITR oligonucleotide probe
A-1 (5'TTGGCCACTCCCTCTCTGCG3') (SEQ ID NO: 4), derived
from A region of ITR, kindly provided by N. Muzyczka)
was performed as follows: the membrane was
prehybridized in 10 ml solution containing 5X SSC, 10X
Denhardt's solution, 10% dextran sulfate and 5% SDS at
60°C for at least 1 hr. 25 ng of ^{32}P end labeled
oligo-probe and 200 ug heat-denatured salmon sperm DNA
in 0.5 ml H$_2$O were added. Hybridization was continued
at 60°C overnight. The membrane was washed twice in
3X SSC and 5% SDS at 60°C for 30 minutes and once in
0.2X SSC at room temperature for 10 minutes. For
Southern blot analysis of lacZ expressing cell clones
a ^{32}P labeled chromosome 19 specific probe was used
(Samulski, R.J. et al., 1991, EMBO J. 10:3941-3950).
To determine whether the CMV/lacZ DNA

fragments had integrated specifically into chromosome
19, high molecular weight genomic DNA was extracted
from transfected cells as described by Sambrook et al.
(1989, Molecular Cloning: A Laboratory Manual. Cold
Spring Harbor Laboratory Press. Cold Spring Harbor,
N.Y.). The genomic DNA was used as template in PCR
reactions designed to amplify junction fragments. The
following PCR primer was used in the PCR reaction.
          5'-GTGAATTGTAATACGACTCACTATAGGGCG-3'.

6.1.3.   PCR AND CONSTRUCTION OF ITR PLASMID
          Low molecular weight DNA from AAV and Ad5
infected cells was used as template for the PCR
reaction with a single primer derived from D-sequence
of AAV. The PCR was performed at 94°C 1 min., 45°C 30
seconds and 72°C 1 min. for 35 cycles in a 50 ul
reaction solution containing 20 mM Tris-HCl (pH8.8),
1.5 mM MgCl, 50 mM KCl, 2.5% formamide, 100 uM dATP,
dCTP and dTTP, 75 uM 7-deazo-dGTP, 25 uM dGTP, 1.5U
AmpliTaq (Perkin Elmer Cetus), 1 ng AAV DNA and 100
pmole primer TR-1 (5'-GGAATTCAGGAACCCCTAGTGATGG3-3')
(SEQ ID NO: 3). The PCR product was purified by
agarose gel electrophoresis, cut with EcoRI and
ligated with an EcoRI cut and dephosphorylated pGEM 3Z
plasmid (Promega). The ligated plasmid was
transformed into *E.coli* Sure strain (Stratagene).
Positive clones named pDD's were screened for the
presence of double-D terminal repeat and confirmed by
dideoxy-sequencing with 7-deazo-dGTP substituted for
dGTP (Sanger, F. et al., 1977, Proc. Natl. Acad. Sci.
USA 74:5463-5467). Subsequently, a neo gene was
cloned into the SalI site of pDD-2 resulting in the
plasmid pDD-neo.

6.1.4.   CLONING OF NEO-RESISTANT CELL LINES
          Ad5 infected 293 cells were cotransfected
with pDD-neo and pAAV/Ad (Samulski, et.al., 1989. J.

Virol. 63:3822-3828) for 48 hrs. The cells were
frozen and thawed three times and then incubated at
56°C for 1 hour to inactivate the Ad5 virus. The cell
lysate containing the DD-neo recombinant AAV virus was
used to infect the human cell line Detroit 6. The
5   cells were inoculated for 24 hours, then selected with
G418 at 400ug/ml to obtain neo-resistant clones.
Various clones were superinfected with wild-type AAV
and Ad5 at a MOI of 10 to rescue the latent neo-AAV.

                    6.2.   RESULTS
10
    6.2.1.   CONSTRUCTION OF ITR WITH DOUBLE D SEQUENCE
            The Polymerase Chain Reaction (PCR) was used
to construct the inverted terminal repeat with a D'
sequence added to the other end. The rationale is
based on the T-shape structure of the ITR. In the
15
first round of PCR reaction, the AAV viral IRT will
self-prime the elongation to produce a long T-shaped
hairpin structure containing D and D' on the stem.
Upon denaturation, this DNA can serve as template for
single-primed PCR.
20
            Because of the high GC content and the
strong palindromic structure in the ITR region,
several strategies such as 7-deazo-dGTP, 2.5%
formamide, and high concentration of primer were
utilized to tackle the PCR problems and yield
25
sufficient desired PCR product. For the convenience
of cloning, an EcoRI recognition sequence was attached
to the 5'of the primer so that the PCR product can be
cut by EcoRI and easily cloned into the polylinker of
pGEM 3Z. Due to the instability of the ITR in
30
bacteria host, the recombinant plasmid was transformed
into an *E.coli* SURE strain (Stratagene) in which the
ITR was rather stable. By using the above strategy,
we obtained numerous positive clones. Some clones
were characterized by restriction digestion and
35
sequencing. One of the clones is shown in Figure 2

- 26 -

bearing an insert of D'ABB'CC'A'D in the EcoRI site of
the pGEM-3Z. This plasmid was named pDD-2 and was
used in the following transfection experiments.

6.2.2.   pDD-2 REPLICATION IS DEPENDENT ON *REP*

          In order to assay the capability for
replication, Plasmid pDD-2 was transfected into Ad5
infected 293 cells with or without cotransfection of a
helper plasmid pAAV/Ad, which contains functional *REP*
and *CAP* genes but without the ITR, so that it can not
replicate. Due to the lack of functional origins this
molecule can only supply *REP* and *CAP* proteins *in
trans*. Post transfection for 48 hours, the plasmid
DNA was extracted and separated on 1% agarose gel with
or without DpnI digestion. DpnI only digests the
input methylated plasmid DNA while leaving the
replicated (demethylated) DNA intact. The results
demonstrated that in the absence of the helper
plasmid, pDD-2 plasmid did not replicate therefore the
DNA is completely DpnI sensitive (FIG. 3, lane 1 and
2). However, in the presence of the helper plasmid,
pDD-2 replicated very efficiently as evidenced by the
resistance to DpnI digestion and the existence of
monomer and dimer molecules: the typical AAV
replication pattern (FIG. 3, lane 3 and 4). The pDD-2
replication is dependent on two factors: the double-D
in cis and *REP* gene products *in trans*, because the
cloning vector pGEM-3Z did not replicate under the
same conditions and a plasmid containing only *REP* gene
without *CAP* gene can also supply the helper function
in trans for pDD-2 (data not shown).

          Since the replication of pDD-2 with one
modified ITR was very efficient, a comparison was made
between pDD-2 and two other infectious AAV plasmids,
psub201 [Samulski, et.al., 1987. J. Virol, 61:3096-
3101.] and pSM620 [Samulski, et.al., 1982, Proc. Natl.
Acad. Sci. U.S.A. 79:2077-2081.], which possesses two

ITRs as well as wild type *REP* and *CAP* genes. The
pDD-2 was cotransfected into Ad5 infected cells with
equal amounts of either pAAV/Ad helper (without ITR),
psub201 or pSM620. The plasmid DNA was extracted 2
days post transfection, digested with DpnI, separated
on 1% agarose gel. Southern blot was performed with
an oligonucleotide probe from the A sequence of the
ITR so that it can detect all the replicated DNA
containing ITRs. As shown in Figure 4, all three
plasmids containing AAV coding genes can complement
the pDD-2 replication equally well. However, psub201
itself replicated at a much lower level although it
can complement pDD-2 replication effectively. pSM201
replicated at a similar level as pDD-2.

In order to determine whether the
effectiveness of pDD-2 replication was due to the
special double-D or due to the smaller size of the
plasmid (2.9 kb), a neo gene fragment of 1.2 kb was
inserted into the SalI site at the polylinker of
pDD-2. The new plasmid pDD-neo is 4.1 kb in size,
close to the size of wild type AAV (4.68 kb). This
plasmid converted from a duplex circular to a linear
molecule and replicated as efficiently as the parental
pDD-2 (FIG. 5). double-D plasmids were constructed
with sizes up to 7.5 kb . These molecules also
efficiently replicate (data not shown). The above
results suggest that the double-D is an excellent
substrate for Rep-dependent replication.

6.2.3. REPLICATION AND RESCUE IS VIA AAV MECHANISM

AAV inverted terminal repeats have been
proven to be the viral replication origins. *In vitro*,
these sequences are recognized as a substrate by *REP*
protein, a site-and-strand-specific nickase and
helicase. ITRs have also been considered as the
substrate for AAV rescue [Muzyczka, N. 1992, Current
Topics in Microbiology & Immunology. 158, 97-129].

Since the double-D plasmids contain one unique ITR and
we have demonstrated that this sequence replicates
only in the presence of *REP* proteins, it is attractive
to predict that the rescue and replication are through
similar AAV rescue and replication mechanisms (FIG. 1,
A and B).

5

      In order to test the above assumption, pDD-2
DNA was transfected into Ad5 infected 293 cells with
or without helper plasmid, or transfected into
uninfected 293 cells. Subsequently, the plasmid DNA
was subjected to restriction analysis by two single

10

cutter enzymes ScpI and ScaI respectively (for map,
see FIG. 2). The DNA was probed with ITR
oligonucleotide so that only the ITR-containing
fragments would be detected. The results are shown in
Figure 6. After SspI or ScaI digestion, a linear full

15

length plasmid band could be observed throughout all
the lanes (P to 6). This band was derived from the
unresolved input circular plasmid. While in lane 1
and 4 (Ad5 plus helper plasmid), four additional bands
with expected molecular weight could also be seen.

20

Two of them arose from internal head-to-head and tail-
to-tail fragments of the digested dimer molecules..
The other two bands are derived by digested monomer
and dimer external fragments, most likely suggesting
that pDD-2 is resolved at the unique double-D site and

25

replicated via AAV replication scheme. It is
noteworthy that in Ad5 infected cells (lane 2 and 5)
and uninfected cells (lane 3 and 6), two fainter bands
from the resolved monomer were also visible,
suggesting that some cellular mechanism can initiate

30

the rescue process at the double-D site in the absence
of any other AAV sequence or AAV gene product.
Although, such rescued DNA could not replicate in the
absence of Rep proteins (see FIG. 3, lane 2) this
suggests that the double D substrate may confer

35

special features involved in the first step of AAV

recognition not seen with the conventional AAV
plasmids containing two wild type ITR's.

6.2.4. ONE DOUBLE-D IN-*CIS* IS SUFFICIENT FOR AAV VIABILITY

Plasmid pDD-neo was used to generate the
DD-neo virus preparation as described in Section
6.1.4.   The cell lysates containing the recombinant
virus particles were then used to infect human Detroit
6 cells.   Two weeks post infection cells were selected
against G418.   A number of neo-resistant clones were
isolated, indicating that the recombinant viruses were
made and transduction was accomplished. DD-neo
resistant cell lines were superinfected with wild type
and AAV-2 and Ad5 and assayed for transduced DNA
rescue and replication. Then the viral DNA was
extracted and probed with a neo gene fragment.
Examples of DD-neo cell lines that rescued DD-neo
viral DNA replicated as monomer and dimer (data not
shown).   These results demonstrated that the 165 bp
single double-D is the only *cis*-sequence required to
fulfill all the steps in AAV life cycle.   Thus, the
processes such as rescue from the plasmid, replication
of the DNA, encapsidation of the virus, infection into
the cells, integration into the chromosome and rescue
back again were all mediated by this unique double D
inverted terminal repeat sequence.

6.2.5. TRANSFECTION OF DOUBLE-D RECOMBINANT VECTORS

Human 293 cells were transfected with either
double-D ITR CMV/LacZ constructs or CMV/LacZ
constructs lacking the double-D sequences.   These
particular constructs contain the lacZ gene which
encodes the E. coli β-galactosidase reporter protein,
under the transcriptional control of the CMV

- 30 -

(cytomegalovirus) constitutive promoter.   In addition,
the CMV/LacZ plasmids were cotransfected with pHIV-
REP, a recombinant plasmid containing the gene
encoding AAV *REP* protein under the transcriptional
control of the HIV promoter (Antoni, B.A. et al.,
1991, J. Virology 65:396-404).

5

Six weeks post-transfection cells were
stained to determine what percent of the cells were
expressing β-galactosidase activity. Detection of
β-galactosidase activity in cell extracts indicates
the presence of chromosomally integrated lacZ

10

constructs. Only the double-D CMV/LacZ constructs
that were cotransfected with a vector capable of
expressing the *REP* protein were found to be expressing
β-galactosidase activity.

Southern Blot analysis of the transfected

15

cells confirmed that those cells expressing
β-galactosidase activity had integrated the double-D
CMV/LacZ constructs into their genome (Fig. 11 and
12). In addition, PCR analysis indicated that the
CMV/LacZ plasmids had integrated into the specific

20

region of chromosome 19 into which wild type AAV
normally integrates (TABLE I).

25

30

35

- 31 -

TABLE I

Cells: TA55, TA67, TA69:

GTGAATTGTAATACGACTCACTATAGGGCGAATTCAGGAACCCCTAGTGATGGAGT

TGGCCACTCCCTCACTGCGCGCTCGCTCGCTCACTGAGGCCGACGGTATCAGCGCC

CTGCACCAGGTCAGCGCCCCCCGC

5   AAV sequence is joined to BamHI fragment of chromosome
           19 at position 1695.

Cells: TA8, TA22, TA65, TA76:

GTGAATTGTAATACGACTCACTATAGGGCGAATTCAGGAACCCCTAGTGATGGAGT

TGGCCACTCCCTCACTGCGCGCTCGCTCGCTCACCGCAGCGAGGCCTGGGCTTTGC

CACCCTATGGT

10   AAV sequence is joined to BamHI fragment of chromosome
           19 at position 1830.

Cells: TA18, TA29:

GTGAATTGTAATACGACTCACTATAGGGCGATTCAGGAACCCCTAGTGATGGAGTT

GGCCACTCCCTCACTGCGCGCTCGCTCGCTCACTGAXGGCTTTGCCACCCTATGGT

15

GACACCCC

AAV sequence is joined to BamHI fragment of chromosome
19 position 1836.

Cell: TA60, TA68:

GTGAATTGTAATACGACTCACTATAGGGCGATTCAGGAACCCCTAGTGATGGAGTT

20   GGCCACCCTATGCTGACACCCCGTCCC

AAV sequence is joined to BamHI fragment of chromosome
19 at position 1843.

Cells: TA16:

25   GTGAATTGTAATACGACTCACTATAGGGCGAATTCAGGAACCCCTAGTGATGGAGTT

GGCCACTCCCTCACTGCGCGCTCGCTCGCTCACCCCCACTTCCGAATTGGAGCCGC

AAV sequence is joined to BamHI fragment of chromosome
19 at position 1891.

Cells: TA45, TA63, TA64, TA70, TA75, TA88:

30   GTGAATTGTAATACGACTCACTATAGGGCGAATTCAGGAACCCCTAGTGATGGAGT

TGGCCACTCCCTCACTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTT

CGCCGACGCCCGGGCTTTGCCCTCTCCTGAACCTGAG

AAV sequence is joined to chromosome 19 at position
2143.

Sequences of Chromosome 19 are underlined.

35

These data indicate the following: (i) that
only *cis*-acting sequence required for targeted
integration by AAV is the double-D sequence and (ii)
the AAV REP protein is sufficient to act *in trans* to
target site specific integration of recombinant vector
5   DNA into genomic host DNA.

The present invention is not to be limited
in scope by the exemplified embodiments disclosed
herein which are intended as illustrations of single
aspects of the invention, and any clones, DNA or amino
10   acid sequences which are functionally equivalent are
within the scope of the invention. Indeed, various
modifications of the invention, in addition to those
shown and described herein, will become apparent to
those skilled in the art from the foregoing
15   description and accompanying drawings. Such
modifications are intended to fall within the scope of
the appended claims.

It is also to be understood that all base
pair and amino acid residue numbers and sizes given
20   for nucleotides and peptides are approximate and used
for the purposes of description. Various publications
are cited herein that are hereby incorporated by
reference in their entireties.

25

30

35

WHAT IS CLAIMED IS:

1.   A purified and isolated nucleic acid
molecule comprising a DNA nucleotide sequence that
        (a)   hybridizes under highly or moderately
               stringent hybridization conditions to
               the sequence depicted in Figure 9 (SEQ.
               ID NO:1); and
        (b)   which is capable of directing the
               replication and assembly into AAV
               various, and/or the integration into
               the host genome, of recombinant DNA
               containing said nucleic acid molecule.

2.   A purified and isolated nucleic acid molecule
comprising a 165 base pair inverted terminal repeat
sequence as depicted in Figure 9 (SEQ ID NO: 1).

3.   A recombinant DNA vector comprising a DNA
nucleotide sequence encoding a protein of interest and
a 165 base pair inverted terminal repeat sequence as
depicted in Figure 9 (SEQ ID NO: 1).

4.   A recombinant DNA vector comprising a DNA
nucleotide sequence encoding a protein of interest and
the nucleic acid molecule of claim 1.

5.   The recombinant DNA vector according to claim
3 or 4 further comprising a DNA nucleotide sequence
that encodes the AAV REP and CAP proteins.

6.   The recombinant DNA vector according to claim
3 or 4 further comprising a DNA nucleotide sequence
recognized by bacterial gamma delta resolvase as
depicted in Figure 10 (SEQ ID NO: 2).

7.   The recombinant DNA vector according to claim
5 further comprising a DNA nucleotide sequence
recognized by bacterial gamma delta resolvase as
depicted in Figure 10 (SEQ ID NO: 2).

8.   A method of replicating and encapsidating a
recombinant DNA molecule into an AAV virion,
comprising:

    a)   culturing a eukaryotic cell containing
      (i) a helper virus, (ii) a recombinant
      nucleic acid encoding AAV REP and CAP
      proteins, and (iii) a recombinant
      nucleic acid that contains a DNA
      sequence of interest and a 165 base
      pair inverted terminal repeat sequence
      as depicted in Figure 9 (SEQ ID NO: 1);
      whereby the nucleic acid containing the
      165 base pair inverted terminal repeat
      sequence and the DNA sequence of
      interest is replicated and assembled
      into AAV virions; and
    (b)   collecting virions produced.

9.   A method of replicating and encapsidating a
recombinant DNA molecule into an AAV particle,
comprising:

    a)   culturing a eukaryotic cell containing
      (i) a helper virus, (ii) a recombinant
      nucleic acid encoding AAV REP and CAP
      proteins, and (iii) a recombinant
      nucleic acid that contains a DNA
      sequence of interest and the nucleic
      acid molecule of claim 1; whereby the
      recombinant nucleic acid containing the
      DNA sequence of interest and the
      nucleic acid molecule of claim 1 is

replicated and assembled into AAV
virions; and

(b)   collecting virions produced.

10.   The method according to claim 8 or 9 in
which the helper virus is an adenovirus.

11.   The method according to claim 8 or 9 in
which the helper virus is a herpes simplex virus.

12.   The method according to claim 8 or 9 in
which the recombinant nucleic acid encoding the AAV
REP and CAP proteins is a plasmid vector comprising:

(a)   promotors which control the expression
of the AAV REP and CAP RNA;

(b)   translation initiation signals for the
REP and CAP mRNA;

(c)   DNA sequences encoding the REP and CAP
proteins; and

(d)   transcriptional termination signals.

13.   The method according to claim 8 in which the
recombinant nucleic acid containing the DNA sequence
of interest and the 165 base pair terminal repeat
sequence is a plasmid vector comprising:

(a)   a promoter;

(b)   a DNA sequence of interest transcribed
under the control of the promoter; and

(c)   a 165 base pair inverted terminal
repeat sequence as depicted in Figure 9
(SEQ ID NO: 1).

14.   The method according to claim 8 in which the
recombinant nucleic acid containing the DNA sequence
of interest and the 165 base pair terminal repeat
sequence is a plasmid vector comprising:

(a)   a promoter;

    (b)   a DNA sequence of interest transcribed under the control of the promoter; and

    (c)   the nucleic acid molecule of claim 1.

15.   A method of replicating and encapsidating a recombinant DNA molecule into a viral particle comprising:

    (a)   propagating a recombinant nucleic acid comprising (i) a recombinant viral vector sequence comprising a gene of interest; (ii) viral genes providing helper functions; and (iii) two bacterial gamma delta resolvase recognition sequences as depicted in Figure 10 (SEQ ID NO: 2) flanking the viral vector sequence;

    (b)   treating the propagated nucleic acid with bacterial gamma delta resolvase; and

    (c)   culturing a eukaryotic cell containing a helper virus and said resolvase treated recombinant nucleic acid; whereby the resolved recombinant DNA is replicated and encapsidated into a viral particle.

16.   A method of replicating and encapsidating a recombinant DNA molecule into an AAV particle comprising:

    (a)   treating a recombinant nucleic acid comprising a 165 base pair terminal repeat sequence as depicted in Figure 9 (SEQ ID NO: 1) and a bacterial gamma delta resolvase recognition sequence as depicted in Figure 10 (SEQ ID NO: 2), with bacterial gamma resolvase enzyme; and

- 37 -

        (b)   culturing a eukaryotic cell containing
               a helper virus and said resolvase
               treated recombinant nucleic acid;
               whereby the resolved recombinant
               nucleic acid is replicated and
               encapsidated into an AAV particle.

        17.   The method according to claim 16 in which
    the helper virus is an adenovirus.

        18.   The method according to claim 16 in which
    the helper virus is a herpes simplex virus.

        19.   The method according to claim 16 in which
    the recombinant nucleic acid containing the bacterial
    gamma delta resolvase recognition sequence is a
    plasmid vector comprising:

        (a)   a bacterial gamma delta resolvase
               recognition sequence;
        (b)   a promoter;
        (c)   a DNA sequence of interest transcribed
               under the control of a promoter; and
        (d)   a 165 base pair inverted terminal
               repeat sequence as depicted in Figure 9
               (SEQ ID NO: 1).

        20.   The method according to claim 16 in which
    the recombinant nucleic acid containing the bacterial
    gamma delta resolvase recognition sequence is a
    plasmid vector comprising:

        (a)   a bacterial gamma delta resolvase
               recognition sequence;
        (b)   a promoter;
        (c)   a DNA sequence of interest transcribed
               under the control of a promoter;

- 38 -

    (d)   a 165 base pair inverted terminal
            repeat sequence as depicted in Figure 9
            (SEQ ID NO: 1); and

    (e)   a DNA nucleotide sequence that encodes
            the AAV REP and CAP proteins.

5

    21.   A method of transferring genetic information
into a eukaryotic cell comprising transfection of said
eukaryotic cell with a recombinant DNA molecule
containing the nucleic acid molecule of claim 1 or 2.

10

    22.   A method of transferring genetic information
into a host comprising:

    (a). encapsulating a recombinant DNA
           molecule containing the nucleic acid

15
           molecule of claim 1 or 2 into a
           liposome; and

    (b)   administering the liposome preparation
           to said host.

20
    23.   The method claim 22 in which a nucleic acid
molecule encoding the AAV REP protein is co-
encapsulated with the recombinant DNA molecule.

    24.   The method of claim 22 in which AAV REP

25
protein is co-encapsulated with the recombinant DNA.

    25.   A stock of recombinant adeno-associated
virus produced according to the method of claim 8
or 9.

30
    26.   A host cell which produces stocks of
recombinant adeno-associated viruses and produced by a
method comprising:

    (a)   culturing said host cell containing (i)

35
           a helper virus, (ii) a recombinant
           nucleic acid encoding AAV CAP and REP

- 39 -

proteins, and (iii) a recombinant
nucleic acid that contains a DNA
sequence of interest and a 165 base
pair inverted terminal repeat sequence
as depicted in Figure 9 (SEQ ID NO: 1);
whereby the nucleic acid containing the
165 base pair inverted terminal repeat
sequence and the DNA sequence of
interest is replicated and assembled
into AAV virions; and

(b)   collecting virions produced.

27.   A eukaryotic cell comprising a recombinant
DNA molecule containing the nucleic acid molecule of
claim 1 or 2.

FIG.1A

FIG.1B

FIG.1C

FIG.2

FIG.3A   FIG.3B

FIG. 4A

FIG. 4 B

FIG.5A   FIG.5B

FIG. 6

FIG.7

FIG.8

```
5 - AGGAACCCCT AGTGATGGAG TTGGCCACTC CCTCTCTGCG CGCTCGCTCG CTCACTGAGG    60
    CCGGGCGACC AAAGGTCGCC CGACGCCCGG GCTTTGCCCG GGCGGCCTCA GTGAGCGAGC   120
    GAGCGCGCAG AGAGGGAGTG GCCAACTCCA TCACTAGGGG TTCCT - 3'                165
```

FIG.9

```
5'- CCCTGTATCC TAAATCAAAT ATCGGACAAG CAGTGTCTGT TATAACAAAA AATCGATTTA    60
    ATAGACACAC CAACAGCATG GTTTTTATGT GTGCGATAAT TTATAATATT TCGGACAGGG - 3'  120
```

FIG.10

FIG.11

13/13

FIG.12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/06786

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00; C12N 7/01, 15/10, 15/86; C07H 21/04
US CL :435/69.1,172.3, 320.1; 536/23.1, 24.1; 424/93.1, 93.6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1,172.3, 320.1; 536/23.1, 24.1; 424/93.1, 93.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis, Medline, Biotech
Search Terms: adeno-associated virus, viral vector, gamma delta resolvase, ITR

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,139,941 A (MUZYCZKA et al.) 18 August 1992, Figures 1-2 and columns 6-7.	1-27
A	KOTIN, Prospects for the Use of Adeno-Associated Virus as a Vector for Human Gene Therapy. Human Gene Therapy. 1994, Vol. 5, pages 793-801, especially pages 798-799 and Figure 2.	1-27
A	SAMULSKI et al. Cloning of adeno-associated virus into pBR322: Rescue of intact virus from the recombinant plasmid in human cells. Proceedings of the National Academy of Sciences. March 1982, Vol. 79, pages 2077-2081, especially page 2079.	1-27

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 AUGUST 1996	04 SEP 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DAVID GUZO
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*